GROWTH AND PHYSIOLOGICAL RESPONSES OF MUGWORT (*Artemisia vulgaris* L.) TO DIFFERENT IRRIGATION FREQUENCIES

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Received date: 01.03.2017 Accepted date: 09.05.2017

ABTRACT

A pot experiment was conducted in a net house to evaluate the effects of three different irrigation frequencies (irrigation at a 7-day interval (T1), a 14-day interval (T2) and a 21-day interval (T3)) on the growth of three mugwort accessions. Results from the experiment revealed that different irrigation frequencies significantly affected the agronomical, anatomical, and physiological characteristics of mugwort. Decreasing the irrigation frequency from a 7-day interval (T1) to a 21-day (T3) led to significant decreases in plant height, leaf number, leaf area, and dry matter accumulation of each mugword accession. The highest value of plant dry weight was recorded in T1G1 (5.98g plant^{-1}) and the lowest was observed in T3G1 (2.53g plant^{-1}). Among the three mugwort accessions used in the study, G6 was seen to have the highest drought tolerance performance, followed by G1 and G7, respectively.

Keywords: *Artemisia vulgaris* L., irrigation frequency, mugwort.

Phản ứng của cây ngải cứu (*Artemisia vulgaris* L.) với các tần suất tưới khác nhau tại Gia Lâm, Hà Nội

TÓM TẮT

Thí nghiệm được tiến hành trong nhà lưới có mái che tại Học viện Nông nghiệp Việt Nam nhằm đánh phản ứng của cây ngải cứu với các khoảng cách tưới khác nhau thông qua một số chỉ tiêu nông học, sinh lý. Công thức xử lý hạn thông qua khoảng cách giữa các lần tưới hạn T1 (1 tuần/lần), T2 (2 tuần/lần), T3 (3 tuần/lần). Kết quả cho thấy tăng dần khoảng cách giữa các lần tưới đã làm giảm chiều cao cây, số nhánh, diện tích lá và khả năng tích lũy chất khô. Trong điều kiện thiếu nước kéo dài, bộ rễ cây ngải cứu có xu hướng tăng số lượng rễ cấp 1, tăng chiều dài và chiều rộng bộ rễ cũng như tăng hàm lượng diệp lục b. Trong điều kiện thiếu hụt nước đã làm giảm số lượng bó mạch rễ của cây ngải cứu. Khả năng chịu hạn của các mẫu giống tham gia thí nghiệm được xếp theo thứ tự G6 > G1 > G7.

Từ khóa: *Artemisia vulgaris* L., hạn, ngải cứu.

1. INTRODUCTION

The practice using of plants as a means of creating medicinal substances has been seen in many civilizations since ancient times, and plays a central role in folk medicine. It has been estimated that 80% of the world's population relies solely on traditional herbs as the source for primary health care (Annon, 2008). Among medicinal plants, mugwort (*Artemisia vulgaris* L.) appears to be one of the most commonly used and has proven itself to have a wide range of medicinal applications. In Chinese medicine, mugwort is used as a means for pain relief, and wound and bronchitis treating. More importantly, recent studies have revealed the effectiveness of using mugwort as tonics, antimalarials, antihelmintics, and antidiabetics (Uzun *et al.*, 2004).

Recently, most of the research carried out in the leaves of mugwort has had aims of evaluating its medicinal effects (Uzun *et al.*,

2004) and chemical composition (Umano *et al*., 2000; Judđentienë and Buzelytë, 2006). Although there is an increasing demand for mugwort for medical materials as well as food, the production of mugwort has been found to be restricted by the cold and dry weather during winter in the north of Vietnam. To date, there exists a lack of understanding in how mugwort responds to water deficit and drought. This experiment aims to assess the growth and physiological responses of three mugwort accessions to different irrigation frequencies. The results from this experiment can provide a basis for developing strategies to maintain stable yields of mugwort in northern Vietnam.

2. MATERIALS AND METHODS

2.1. Plant materials

Three mugwort accessions collected in the north of Vietnam (G1, G6 and G7) were used in this experiment. Brief morphological descriptions of each accession are given in Table 1.

2.2. Experiment design

Three irrigation frequencies, at 7-day (T1), 14-day (T2), and 21-day (T3) intervals, and 3 mugwort accessions (G1, G6, G7) were employed in this study (Table 2). A 3 x 3 factorial experiment (9 treatments) was carried out following a split plot design with 3 replications (20 pots were counted as a replication). Mugwort seedlings (with 2 leaves and 10cm in height) were transferred into pots (18 x 20 x 25cm) containing 3kg of alluvial soil. Each pot was irrigated with 300ml of tap water.

2.3. Measurements

Leaf wilting (%) and recovery (%) of mugwort were measured at 3 and 7 days after re-irrigating.

At the 120th day of the experiment, 12 plants from each treatment were collected for the measurement of growth parameters, including: stem height (cm), no. of leaves plant^{-1}, no. of branches per plant^{-1}, stem diameter (cm), leaf area (dm^2 plant^{-1}), no. of adventitious roots, adventitious root diameter (cm), root length (cm), root width (cm), and dry weight accumulation (g plant^{-1}).

Chlorophyll a content (mg g^{-1}), chlorophyll b content (mg g^{-1}), and carotenoid content (mg g^{-1}) were measured followed Arnon (1949).

Anatomical parameters were observed and measured in plants on the 120th day of the experiment using the double staining method (Methylene Blue and Carmine). The no. of bundle sheathes in each root was recorded.

Table 1. Brief morphological descriptions of 3 mugwort accessions

Accession	Color			Hairiness			Origin
	Stem	Leaf blade	Leaf Vein	Stem	Upper leaf surface	Lower leaf surface	
G1	Purple	Green	Purple	None	None	None	Thuan Chau- Son La
G6	Purplish green	Green	Green	Very dense	Very dense	Very dense	Thai Thuy-Thai Binh
G7	Purplish green	Green	Green	Very dense	Slight	Very dense	Thuan Chau-Son La

Table 2. Nine treatments used in the study

Accession	Irrigation frequency		
	T1 (irrigation at 7-day interval)	T2 (irrigation at 14-day interval)	T3 (irrigation at 21-day interval)
G1	T1G1	T2G1	T3G1
G6	T1G6	T2G6	T3G6
G7	T1G7	T2G7	T3G7

2.4. Data analysis

All data collected in the study were subjected to Analyses of Variance (ANOVA) using Microsoft Excel and Cropstat (version 7.2).

3. RESULTS AND DISCUSSIONS

3.1. Effects of irrigation frequency on leaf-wilting and recovery of mugwort

One of the early responses to drought developed by plants is the dehydration of water in the leaves. Besides, a water deficit in the leaf surface leads to the loss of water in guard cells together with a decline of turgidity and thus, makes stomata pores close (Hightshoe, 1987). Dehydration and loss of water in leaves finally results in leaf wilting. Levels of leaf wilting vary and can be grouped into: incipient (i), temporary (ii), and permanent (iii) (Hightshoe, 1987). While incipient wilting doesn't lead to leaf drooping, temporary wilting causes leaf drooping during the day and recovery during night time. Prolonged and sustained drought can make leaves permanently wilted and unable to recover during night time. Leaf recovery from permanent wilting therefore, needs thorough re-irrigation of the soil (Hightshoe, 1987). In addition, permanent leaf wilting is believed to have many adverse effects on different aspects of plant growth including photosynthesis, respiration, and leaf transpiration (Athar and Ashraf, 2005). Early drought responses can be seen by observation of leaf wilting and recovery (recorded at 3 and 7 days after re-irrigation).

Data regarding mugwort leaf wilting and recovery are presented in Table 3. Among the three irrigation frequencies, plants grown under T3 (irrigation at a 21-day interval) obtained a higher rate of leaf wilting and lower recovery rate compared to plants grown under T1 and T2. Under T3, the highest value of leaf wilting was recorded in G1 (100.00%) followed by G7 (60.00%) and G6 (46.67%), respectively. However, compared to other plants, mugwort is believed to be relatively good at recovering from wilting. This can be explained by the fact that mugwort has white wooly hairs coating the leaf surface which helps to reflect heat and hence, reduce the loss of water in the leaves (El-Sahhar, 2010). This allows the leaves of mugwort to be able to withstand different levels of drought and maintain the plant's recovery. According to Roy *et al.*, (1999), leaf hairs play an important role in ameliorating the effects of imposed drought. An increase in leaf hair density reduced water loss from the leaf surface while a decrease in leaf hair density was believed to increase light reception (Roy *et al.*, 1999). Among the three mugwort accessions in this experiment, G6 showed highest drought tolerance potential. At 7 days after re-irrigation, under T3, G6 obtained the highest rate of recovery from wilting (80.00%), followed by G1 and G7 (66.67%). This may be mainly due to the dense layer of hairs on both the upper and lower leaf surfaces in G6. Compared to G1 and G7, G6 plants have the highest leaf hair density. Having dense hairs coated on both sides of the leaf allows G6 to highly reduce water loss from the leaf surface during prolonged water stress and hence, helps the plant to recover from wilting. This can be seen as one of the mechanisms employed by plants to better adapt to sustained water deficit and drought.

3.2. Effects of different irrigation frequencies on stem and leaf development of mugwort

Plants have developed various mechanisms to cope with water stress both at the cellular and whole-organism level (Farooq *et al.*, 2011). The effects of drought and water stress on plants are varied and can be detected by both morphological and molecular changes. It has been reported that drought and water stress led to reduced leaf size, decreased stem growth and root expansion, increased hair density on leaves and stems, and altered plant and water relations (Farooq *et al.*, 2011). Morphological and growth responses of mugwort to different irrigation frequencies are presented in Table 4.

Table 3. Effects of different irrigation frequencies on leaf wilting and recovery

Irrigation frequency	Accession	Leaf wilting (%)	Recovery (%)	
			3 days after re-irrigation	7 days after re-irrigation
T1	G1	13.33	100.00	100.00
	G6	6.67	100.00	100.00
	G7	6.67	100.00	100.00
T2	G1	33.33	80.00	100.00
	G6	13.33	100.00	100.00
	G7	20.00	66.67	100.00
T3	G1	100.00	53.33	66.67
	G6	46.67	60.00	80.00
	G7	60.00	55.56	66.67

Table 4. Effects of different irrigation frequencies on stem and leaf development of mugwort

Irrigation frequency	Accession	Main stem height (cm)	No. of branch plant^{-1}	No. of leaves plant^{-1}	Leaf area (dm^2 plant^{-1})
T1	G1	26.47	6.68	31.55	12.27
	G6	35.13	6.01	28.84	10.65
	G7	24.69	7.33	24.22	11.44
T2	G1	28.20	5.69	22.89	8.60
	G6	28.40	5.74	24.27	4.65
	G7	21.63	6.26	17.71	7.31
T3	G1	21.58	4.30	13.20	6.89
	G6	24.41	5.13	21.02	4.20
	G7	17.78	4.72	17.20	4.15
$LSD_{0,05\ G*T}$		1.70	1.15	3.32	0.93
Mean T	T1	28.76	6.67	28.20	`11.45
	T2	26.08	5.89	21.26	6.85
	T3	21.26	4.72	17.14	5.08
$LSD_{0,05\ T}$		1.57	0.46	1.47	0.61
Mean G	G1	25.42	5.56	22.55	9.25
	G6	29.31	5.63	24.71	6.50
	G7	21.37	6.10	19.71	7.63
$LSD_{0,05\ G}$		0.98	0.67	1.92	0.54
CV% $_{G*T}$		3.8	11.3	8.4	6.7

Stem height is considered a variable trait and its expression is strongly influenced by environmental and technical factors (Zecevic *et al.*, 2008). According to Ninh Thi Phip *et al.*, (2015), in well-watered conditions, G6 has longer stem internodes compared to G1 and G7 and hence, often obtains greater values for stem height. In this study, different irrigation frequencies significantly affected stem growth and leaf development of mugwort. A lower frequency irrigation (T3) significantly decreased the stem height, number of branches plant^{-1}, number of leaves plant^{-1}, and leaf area of mugwort. The main value of stem height of

mugwort was reduced from 28.76 (cm) in plants grown under T1, to 26.08 (cm) in T2, and was lowest at T3 (21.26 cm). Among the three accessions, the highest stem height was observed in G6 (29.31 cm), followed by G1 (25.42 cm) and G7 (21.37 cm), respectively.

Morphological adaptations of plants to drought and water stress have been reported in previous studies (Wu *et al.*, 2008; Kadiođlu *et al.*, 2012). Leaf adaptation is considered one of the most important factors favoring the success of a plant under drought and poorly-watered conditions. Statistical analysis (p=0.05) revealed significant differences among mugwort accessions and irrigation frequencies in this study. Among the three irrigation frequencies, the lowest leaf number was observed in plants grown under T3 (17.14 leaves plant^{-1}), followed by T2 (21.26 leaves plant^{-1}) and T1 (28.20 leaves plant^{-1}), respectively. The largest reduction in leaf area was also recorded in plants grown under T3 (5.08 dm^2 plant $^{-1}$), followed by T2 (6.85 dm^2 plant $^{-1}$) and T1 (11.45 dm^2 plant $^{-1}$). Water stress caused significant reductions in leaf number and leaf area (LA) of mugwort, confirming the same results as previous studies in almonds (Zamani *et al.*, 2002; Khosroshahi *et al.*, 2014), peaches (Rieger *et al.*, 2003), and apples (Liu *et al.*, 2012). The reduction of leaf area can be seen as an important stress avoidance strategy and is considered the first defensive mechanism employed by plants to withstand different levels of drought (Khosroshahi *et al.*, 2014). Besides, depending on drought duration and intensity, a plant might minimize transpirational water loss by reducing its leaf number. Because individual leaf size was not affected by drought and water stress, the reduction of leaf area (LA) in mugwort is mainly caused by leaf abscission and reduced leaf number (Khosroshahi *et al.*, 2014).

3.3. Effects of different irrigation frequencies on root development of mugwort

The mugwort root system is characterized by light brown rhizomes (up to 1cm in diameter), branching at nodes, mostly distributed in the upper 20cm of the top soil. The rhizomes, together with adventitious roots developed from the nodes of each rhizome, form a complex and extensive underground structure (El-Sahhar, 2010). Root growth and development is strongly affected by drought and water stress. In addition, an extensive root structure is believed to be advantageous for plant growth under drought (Anjum *et al.*, 2011). Besides, root development is believed to enhance water uptake and together with higher proline content, helps the plants maintain a suitable osmotic pressure for survival and growth under drought stress (Djibil *et al.*, 2005). Reponses of mugwort to different irrigation treatments with regard to root growth and development in this study are presented in Table 5.

Data from Table 5 revealed that different irrigation frequencies significantly affected the root characteristics of mugwort (no. of adventitious root plant^{-1}, adventitious root diameter, root length, and root width). Among the three irrigation frequencies, the highest number of adventitious roots was found in plants grown under T3 (57.58 plant^{-1}), followed by T2 (49.92 plant^{-1}) and T1 (46.50 plant^{-1}), respectively. In addition, the highest values for root length and root width were also recorded in plants grown under T3. However, within the same irrigation frequency, no significant differences (p=0.05) in root characteristics (no. of adventitious root plant^{-1}, adventitious root diameter, root length, and root width) were found among the three mugwort accessions. Water stress triggered the development of the mugwort root system by increasing the root length, increasing the number of adventitious roots, and reducing their diameter. Having a smaller diameter allows the adventitious roots to deeply penetrate into smaller soil pores (Franco, 2011) and thus, optimizes water uptake by the root system. This may be considered a key role for mugwort survival and growth under water stress.

Table 5. Effects of irrigation frequencies on root development of mugwort

Irrigation frequency	Accession	No. of adventitious root plant^{-1}	Adventitious root diameter (cm)	Root length (cm)	Root width (cm)
T1	G1	43.50	0.17	8.10	8.58
	G6	46.75	0.22	7.45	7.85
	G7	49.25	0.19	11.89	9.19
T2	G1	44.50	0.16	13.45	13.00
	G6	51.75	0.16	10.90	12.45
	G7	53.50	0.17	9.18	10.23
T3	G1	54.25	0.14	14.15	13.35
	G6	58.50	0.15	13.97	14.15
	G7	60.00	0.16	14.76	13.88
$LSD_{0,05\ G*T}$		4.32	0.06	1.07	1.42
Mean T	T1	46.50	0.19	9.15	8.54
	T2	49.92	0.16	11.18	11.89
	T3	57.58	0.15	14.29	13.79
$LSD_{0,05\ T}$		2.30	0.03	1.46	0.50
Mean G	G1	47.42	0.15	11.90	11.64
	G6	52.33	0.18	10.77	11.48
	G7	54.25	0.17	11.94	11.10
$LSD_{0,05\ G}$		2.50	0.03	0.62	0.82
$CV\%_{\ T*G}$		4.7	1.6	5.2	7.0

Compared to shoot growth, root growth is less influenced by drought and a decrease in the shoot:root ratio can be seen as an early response of plants under drought conditions (Franco, 2011). While shoot growth is rapidly reduced when plants are grown under low water potentials, roots maintain the ability to elongate when being subjected to low water potentials which totally inhibit the growth and development of the shoot (Wu and Cosgrove, 2000). In fact, root architecture (root structure and root distribution) contributes a larger part in determining drought tolerance than root quantity (Farooq *et al.*, 2011). Breeding for a deep and extensive root structure of mugwort therefore, is highly recommended to reduce the effects of drought on its growth and yield.

3.4. Effects of different irrigation frequencies on photosynthetic pigments and dry weight accumulation of mugwort

Drought has been reported to cause a reduction in the content of photosynthetic pigments such as chlorophyll (chlorophyll *a* and chlorophyll *b*) and carotenoids (Mafakheri *et al.*, 2011; Ashraf and Harris, 2013). Besides, severe drought leads to deterioration of thylakoid membranes (Anjum *et al.*, 2011). Changes in photosynthetic pigments have important implications for drought tolerance in plants (Jallel *et al.*, 2011).

While chlorophyll *a* (Chl *a*) content slightly decreased from 0.31 mg/g (T1) to 0.30 mg/g (T2 and T3), a considerable decline of chlorophyll *b* (Chl *b*) was observed across different irrigation frequencies. Chl *b* content declined from 0.74 mg/g (in T1) to 0.73 mg/g (in T2) and 0.64 mg/g (in T3). The reduction of photosynthetic content in leaves under drought has also been reported in wheat (Ashraf *et al.*, 1994), canola (Din *et al.*, 2011), cotton (Massacci *et al.*, 2008), and sunflower (Kiani *et al.*, 2008). The decline of chlorophyll content under drought and a water deficit is mainly due to the damaged chloroplasts caused by the accumulation of active oxygen species (ROS) such as hydroxyl

radicals (OH), hydrogen peroxide (H_2O_2), alkoxy radicals (RO), and anion radicals (O^{-2}) (Farooq *et al.*, 2009). ROS is believed to react with lipids and proteins, leading to destructive damage such as lipid peroxidation and chlorophyll bleaching (Terzi *et al.*, 2006). Since Chl *b* is more sensitive to drought than Chl *a*, a greater decline in Chl *b* is commonly observed in plants (Farooq *et al.*, 2009), and thus, leads to a rise in the Chl *a/b* ratio.

It's well reported that plants have developed both enzymatic and non-enzymatic defense mechanisms to alleviate and reduce damage caused by ROS. This is termed as antioxidant defense and can be seen as one of the important criteria for the screening of drought tolerant genotypes (Faize *et al.*, 2011). Among photosynthetic pigments, carotenoids have an additional role in protecting leaves from oxidative damage caused by ROS. Carotenoids are known to carry the function of photoprotectants by quenching ROS or dissipating heat of excess light energy (McElroy and Kopsell, 2009). Having a high content of carotenoids help the plants to better withstand the adverse effects caused by drought and water deficit. This is entirely consistent with the mechanism of mugwort to tolerate drought when the carotenoid contents recorded in plants with T2 or T3 (0.32 and 0.30mg/g, respectively) were higher than that found in T1 (0.28mg/g). A decline in carotenoid content in plants from T2 to T3 suggested that T2 is the threshold for drought tolerance of mugwort. Less frequent irrigation (than T2) or more severe drought can lead to disorders in metabolic processes and then further restrict the growth of mugwort.

Table 6. Effects of different irrigation frequencies on photosynthetic pigments and dry weight accumulation

Irrigation frequency	Accession	Pigment content (mg/g)			Dry weight (g plant^{-1})
		Chlorophyll *a*	Chlorophyll *b*	Carotenoid	
T1	G1	0.31	0.76	0.28	5.98
	G6	0.31	0.74	0.27	5.21
	G7	0.31	0.72	0.27	5.23
T2	G1	0.30	0.60	0.26	4.18
	G6	0.28	0.95	0.29	5.79
	G7	0.31	0.65	0.41	3.57
T3	G1	0.30	0.65	0.34	2.53
	G6	0.29	0.58	0.29	3.22
	G7	0.30	0.70	0.27	2.92
$LSD_{0,05\ T*G}$		-	-	-	0.34
Mean T	T1	0.31	0.74	0.27	5.47
	T2	0.30	0.73	0.32	4.51
	T3	0.30	0.64	0.30	2.89
$LSD_{0,05\ T}$		-	-	-	0.67
Mean G	G1	0.30	0.67	0.28	4.23
	G6	0.29	0.76	0.26	4.74
	G7	0.31	0.69	0.31	3.91
$LSD_{0,05\ G}$		-	-	-	0.20
$CV\%_{\ T*G}$		-	-	-	4.4

Table 7. Effects of different irrigation frequencies on root anatomy

Irrigation frequency	Accession	Number of bundle sheath		Total number (bundle root^{-1})
		Large bundle (bundle root^{-1})	Small bundle (bundle root^{-1})	
T1	G1	45	43	88
	G6	25	38	63
	G7	50	30	80
T2	G1	41	35	76
	G6	25	30	55
	G7	36	42	78
T3	G1	30	28	58
	G6	20	20	40
	G7	33	18	51
Mean T	T1	40.00	37.00	77.00
	T2	34.00	35.67	69.67
	T3	27.70	22.00	49.67
Mean G	G1	38.67	35.33	74.00
	G6	23.33	29.33	52.67
	G7	39.67	30.00	69.67

Data collected in the experiment also revealed the effects of drought on dry weight accumulation of mugwort. Drought significantly reduced the dry weight of mugwort from 5.47g plant^{-1} in T1 to 4.51 g plant^{-1} in T2 and 2.89 g plant^{-1} in T3. Within the same T3 treatment, G6 appeared to have better tolerance performance with the highest plant dry weight (4.74 g plant^{-1}), followed by G1 and G7 with 4.23 and 3.91 g plant^{-1}, respectively.

3.5. Effects of different irrigation frequencies on root anatomy

Water stress is well known to markedly affect anatomical features in different plant species. It significantly decreased leaf thickness, leaf hair number, xylem vessel area, and vascular bundles (Aldesuquy, 2013). Besides the structural alterations caused in xylem and phloem areas, water stress also leads to reduced xylem conductivity. In addition to the anatomical alterations in leaves, changes in root anatomy provide a deeper understanding of drought tolerance mechanisms in plants.

Data from this experiment revealed that a decrease in irrigation frequency led to decrease in the number of large and small vascular bundles and also in the total number of vascular bundles in the roots of the three mugwort accessions. Mean number of vascular bundles decreased from 77.00 bundles (in T1) to 69.67 bundles (in T2) and was lowest at 49.67 bundles (in T3). The number of vascular bundles varied across irrigation frequencies and mugwort accessions. Among the three accessions in this study, G1 appeared to have the highest number of vascular bundles under water stress (74.00 bundles root^{-1}), followed by G7 (69.67 bundles root^{-1}) and G6 (52.67 bundles root^{-1}).

4. CONCLUSIONS

Water stress significantly affected the agronomical, anatomical, and physiological characteristics of mugwort. A decrease in irrigation frequency from a 7-day interval (T1) to a 21-day (T3) led to significant decreases in plant height, leaf number, leaf area, and dry matter accumulation of each mugwort accession. The highest value of plant dry weight was recorded in T1G1 (5.98g plant^{-1}) and the lowest was observed in T3G1 (2.53g plant^{-1}). Among the three mugwort accessions in the study, G6 was seen to have the highest drought tolerance performance, followed by G1 and G7, respectively. Mugwort adapts to water stress by increasing the number of adventitious root,

having a prolific root system, and maintaining its chlorophyll *a* content while increasing its chlorophyll *b* content.

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NITROGEN WASTE IN THE EFFLUENT FROM AN INTENSIVE SHRIMP FARM AND THE REMOVAL EFFECTIVENESS OF A WASTWATER TREATMENT SYSTEM INTEGRATING SEWEED PRODUCTION

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Received date: 08.03.2017 Accepted date: 04.08.2017

ABSTRACT

Environmental concerns and limitations in water sources for aquaculture production are critical factors in the pursuit of wastewater treatment systems for sustainable aquaculture development. In the study, the accumulation of nitrogen components in the effluent from shrimp ponds was examined and the removal efficiency of a wastewater treatment system were evaluated for a commercial production scale. The wastewater treatment system consisted of three steps: (1) sedimentation, (2) sand-filtration, and (3) bioremediation of a high rate algal pond (HRAP), which cultivated *Ulva ohnoi* seaweed as a monoculture. The study showed that the discharged water from intensive shrimp ponds at the end of the production cycle contained high levels of nitrogen components. Compared to the inlet water (from reservoirs), the total nitrogen (TN) concentration in the effluent increased from 0.26 mg/l to 6.23 mg/l and was composed of 25% total particle nitrogen (TPN), 42% total ammonia nitrogen (TAN), 30% dissolved organic nitrogen (DON), and a very low proportion (approximately 3%) of oxidised nitrogen compounds (NO_x). The combined treatment system effectively removed 78% of TN in the wastewater, reducing TN from 6.23 to 1.33 mgN/l. The removal efficiencies of the sedimentation pond, sand-filtration, and HRAP were 47%, 15%, and 16% TN, respectively. While the sedimentation pond mainly cleared the TPN components; the sand-filtration effectively reduced TAN and converted this compound into NO_x due to its function as a habitat for nitrifying bacterial growth, and the treatment step with HRAP significantly removed both TAN and NO_x in the effluent. Therefore, the treatment system integrating seaweed cultivation presented a high efficiency in removing the nitrogen components in the wastewater from intensive shrimp farms.

Keywords: HRAP, nitrogen components, sand-filter, seaweed, sedimentation, wastewater

Thành phần dinh dưỡng nitơ trong nước thải từ hệ thống nuôi tôm thâm canh và hiệu qủa làm sạch môi trường của hệ thống xử lý nước thải có sự kết hợp của sản xuất rong biển

TÓM TẮT

Thiết kế hệ thống xử lý nước thải từ ao nuôi hiệu quả và tái sử dụng nguồn nước sau khi xử lý là yêu cầu cấp thiết, đặc biệt đối với hệ thống nuôi tôm công nghiệp do những mỗi quan tâm tăng lên về ô nhiễm môi trường và giới hạn nguồn nước sạch cho nuôi trồng thủy sản. Nghiên cứu được thực hiện nhằm xem xét sự tích lũy nitrogen trong nước thải từ hệ thống nuôi tôm công nghiệp và đánh giá khả năng làm sạch của hệ thống xử lý nước thải với quy mô xử lý cho trại sản xuất công nghiệp. Hệ thống xử lý nước thải sử dụng trong nghiên cứu gồm ba bước xử lý: (1) xử lý lắng, (2) lọc cát, và (3) khả năng làm sạch sinh học của ao nuôi rong biển hiệu suất cao (HRAP) với quy mô xử lý cho trại sản xuất công nghiệp. Kết quả nghiên cứu cho thấy, nước thải từ ao nuôi tôm thâm canh về cuối chu kỳ nuôi đã có sự tích lũy ở mức độ cao của hầu hết các hợp chất chứa nitơ. So với nước đầu vào (nước ao chứa) giá trị tổng nitơ (TN) đã tăng từ 0,26 mgN/l lên 6,23 mgN/l với thành phần bao gồm: 25% nitơ ở dạng hạt (total particle nitrogen - TPN), 42% ở dạng tổng ammonia (total ammonia nitrogen - TAN), 30% ở dạng nitơ hữu cơ hòa tan

(dissolved organic nitrogen - DON) và tỷ lệ rất nhỏ (khoảng 3%) thành phần nitơ đã được oxi hóa (NO_x). Hệ thống xử lý nước thải kết hợp đã loại bỏ được 78% TN trong nước thải, làm giảm lượng từ 6,23 xuống 1,33 mgN/l với khả năng làm sạch của 3 bước xử lý bằng ao lắng, ao lọc cát, và rong biển tương ứng là 47%, 15% và 16% TN. Hệ thống ao lắng cho thấy hiệu quả đặc biệt trong việc làm sạch nhóm hợp chất nitơ dạng hạt (TPN), hệ thống lọc cát có tác dụng chủ yếu lên việc chuyển hóa ammonia sang dạng NO_x nhờ chức năng là nơi cư trú cho nhóm vi khuẩn nitrate hóa, trong khi bước xử lý bằng rong biển cho thấy đặc biệt hiệu quả trong việc làm sạch cả 2 nhóm nitơ vô cơ dạng hòa tan TAN và NO_x. Như vậy, hệ thống xử lý kết hợp rong biển cho thấy hiệu quả rất cao trong việc xử lý các nhóm hợp chất nitơ trong nước thải.

Từ khóa: Ao lắng, ao lọc cát, HRAP, nước thải, thành phần nitơ.

1. INTRODUCTION

Aquaculture is one of the most rapidly growing food sectors globally (FAO, 2012), with an annual growth rate from 6 - 10% over the last 30 years (Msangi *et al.*, 2013). The contribution of aquaculture to the world aquatic production (e.g. finfish, crustacean, molluscs) reached 43.1% in 2013, up only 30.6% from a decade ago in 2003 (FAO, 2015). Without a doubt, aquaculture will continue playing an important role in the global supply of aquatic products in the future. Therefore, the technologies for sustainable growth, both economically and environmentally, are of increasing interest.

Along with the increase in production, the farming systems have gradually changed from extensive traditional to semi-intensive and intensive culture systems (Anh *et al.*, 2010; Lebel *et al.*, 2010). The shift is due to both increasing demand for aquaculture products and the improvements in culture techniques (Zhong *et al.*, 2011). For intensive shrimp farming, feeds account for more than half of the production cost (Preston *et al.*, 2001; Thakur & Lin, 2003; Sahu *et al.*, 2013). However, shrimp can convert only around 20 - 30% of the total nitrogen and 10 - 15% total phosphorous from the feeds into their biomass (Funge-Smith & Briggs, 1998; Preston *et al.*, 2001; Thakur & Lin, 2003; Khoi & Fotedar, 2011). The major nutrient proportion (more than 70%) is retained in sediment and the water column of the culture system through feed waste, prawn excretion, and faeces production. The resulting environmental impacts from untreated effluent have raised concerns about the ongoing sustainability of shrimp farming, and the treatment of this waste are important for this ends (Jones *et al.*, 2002). Thus, an efficient wastewater treatment system is an urgent requirement to purify the wastewater before discharging into the environment or to reuse the treated water for aquaculture production.

Understanding the nitrogen components of the wastewater is a prerequisite step to designing an efficient water treatment system. Total nitrogen (TN) can be divided into total particle nitrogen (TPN) and total dissolved nitrogen (TDN) which is comprised of dissolved organic nitrogen (DON) and dissolved inorganic nitrogen (DIN). However, there is a large variation in the total nitrogen concentration and the contribution of each nitrogen component in the wastewater in the culture system. Sedimentation ponds have been commonly used in various countries to treat wastewater from intensive shrimp farms (Preston *et al.*, 2001; Castine *et al.*, 2013). Although the sedimentation step can settle the large-sized particles down due to gravitation, the maximum removal efficiency was no more than 60% of the total nitrogen in the wastewater (Preston *et al.*, 2001; Jackson *et al.*, 2003a; Castine *et al.*, 2013). The sedimentation ponds were less effective in removing small particles and dissolved nitrogen fractions in the wastewater. For recycling the treated water and dealing with the stricter regulations on discharging effluent into the natural environment, a combined treatment system is recommended.

This study aimed to examine the build-up of nitrogen compounds in the effluent from an intensive shrimp farm in Australia and

evaluate the removal efficiencies of a three step combined wastewater treatment that included sedimentation, sand-filtration, and seaweed purification on nitrogen components in the effluent.

2. MATERIALS AND METHODS

2.1. Study location and shrimp farming system

The wastewater treatment system was set up for a commercial scale at Pacific Reef Fisheries, Queensland, Australia, which is adjacent to the Great Barrier Reef. Sea water from the Coral Sea was pumped into a reservoir before being delivered into the shrimp ponds through earthen channels (Fig. 1).

The shrimp farm is comprised of 68 earthen ponds, one ha each, with an average water depth of 1.4 m. Production is classified as moderately intensive: stocking densities were between 32 and 35 P15/m^2, and four to six electric aerators (2 hp) were operated continually in each pond. At the time of the study, the farm production cycle was unsynchronized (i.e., crop age varied between ponds, and production continued year-round). Each pond completed between one and two production cycles per year. Of these, three ponds, which were stocked at the same time, were selected for water sampling. These ponds were 130 - 135 days into their production cycle with shrimp sizes of 31 - 35 grams/shrimp on average at the first water sampling batch. The tiger shrimps were fed 45% protein pellets using a feeding regime of four times daily. The yield of these ponds at harvest were approximately 4.5 to 5 tones of shrimp per hectare.

Little or no water exchange was done for the first 1 - 2 months after a pond was first filled and stocked. Thereafter, water in each pond was partially exchanged at regular intervals of 7 days with an exchange amount of 20% weekly. No fertilizer was added to the ponds during the study. The trial was monitored for a production cycle of 6 months. The salinity of the shrimp pond effluent over the study period varied from 29 - 32 ppt with an average of 30.7 pp. The temperature was measured continually using automatic temperature measurements and a temperature logger. The lowest temperature was taken at 6am and the highest value was taken at 2 pm. During the study period, the lowest temperature was 19.7 and the highest temperature was 31.8°C. A mean value of 7.74 pH was observed for the shrimp effluent.

Figure 1. Location of the shrimp farm

Source: Google maps

2.2. Wastewater treatment system

The wastewater treatment system was designed with three treatment steps as indicated in the Figure 2, including:

Step 1: The first treatment step was targeted to remove the particulate nitrogen fraction by using sedimentation ponds. The effluent from intensive shrimp ponds was drawn into a sedimentation pond and kept in this pond for 3 to 5 days before going to the next treatment step. The shrimp farm is currently using a sedimentation pond with an area of about 2ha for a primary treatment of wastewater before discharging into the environment.

Step 2: The next treatment step, sand filters (SF), was designed to aid the sedimentation pond in the capture of particulates of all size fractions, mainly suspended and fine solid. SF functions also included improved water clarity to *Ulva* growth in the next treatment step, and was also expected to be a bio filter media for the inhabitation and development of the bacteria community to convert organic nitrogen to inorganic forms and among inorganic forms. There are two parallel 120 m length sand-filters constructed at the farm, (SF1: Area = 2,897 m^2; SF2: Area = 3,460m^2) and both contain sand media at a depth of 0.5 m . During the study period, only sand-filter 1 (2,897 m^2) was online. The water depth of the sand-filter was 0.4 - 0.6 m, which was the optimal values reported by the farm (unpublished document). Sand filters were supplied at their maximum flow rates (19 - 23 L/s) and drained at a rate that allowed for maintenance of water levels.

The performance of the sand-filter can reduce over time as the results of filamentous algae and microalgae at the SF surface, particularly at lower water levels. These issues can be detected by periodic measurement of maximum SF filtration rate (discharge valve 100% open and assuming pipework sizing is not the flow restriction) which indicates the need for maintenance and swapping of the SF (preventative and proactive maintenance). Maintenance of sand-filters can be carried out manually or using machines.

Step 3: The last step of treatment aimed to clean up the dissolved inorganic nitrogen components (DIN) using seaweed. The wastewater after the sand-filter treatment step was drawn into a High Rate Algal Pond (HRAP) at a controled flow rate. In the study, *Ulva ohnoi* seaweed was cultivated as a monoculture in the HRAP with a dimension of 150m × 10m (LxW) and a water depth of 50 cm at the centre point to uptake the DIN compounds. The stocking density was maintained at 3 - 4 gram fresh weight/l (FW/l), which was the suitable stocking density reported by the farm (unpublished report). Typically, the *Ulva* more than doubled its mass in 1 week, therefore, seaweed biomass was havested weekly to keep a constant density in the system over time. Paddlewheel rpm settings were fixed and sufficiently high to ensure algae suspension regardless of water depth and algae density. No additional nutrients or additives were added for maintenance of the *Ulva* stock into the SF or HRAP system during the study period. Water exchange rate was at 300% x 24h through the HRAP. During the study, the treated water after going through the HRAP step was discharged into the environment and wasn't used for recycling.

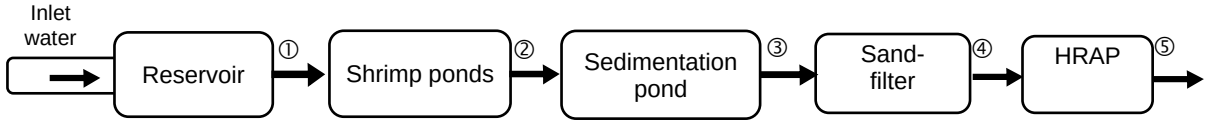

Figure 2. The water flow across the farm and the treatment steps used in the study

Note: The inlet water from the ocean was pumped into a reservoir before being delivering to the culture ponds. The effluent from the shrimp ponds was sent through three treatment steps including sedimentation ponds, sand-filer bed, and high rate algal ponds (HRAP)

Figure 3. Sand-filter bed used in the study

Note: The size of the sand filter bed was 120 m x 24.5 m (L x W) containing sand media at a depth of 0.5 m

Figure 4. High rate algal pond (HRAP) culturing *Ulva ohnoi* as an monoculture

Note: The size of the HRAP was 100 m x 10 m (LxW) with 0.5 m being the deepest point at the center

2.3. Water sampling site and analysis

The water samples were collected from five sources across the farm as indicated in Figure 2 to evaluate the changes in the concentrations of water environmental factors and removal efficiencies of each treatment step. Five sampling batches were conducted during the period from April to May, 2016. For each sampling batch, three samples from each water source were collected with the sampling schedule indicated in Table 1. Water samples from the reservoir and shrimp ponds were collected on the days of water exchange for the shrimp ponds. The discharged water from the shrimp ponds was drawn into the sedimentation pond and left for 3 days of settling. Thus, the samples from the settlement ponds, SF, and HRAP were taken 3 days thereafter. After sampling, the water samples were preserved at approximately 4^0C (using ice boxes) and analysed within 24 hours. The type of nitrogen variables and the methods for analysing and calculating of each of the factors are presented in Table 2.

Table 1. Sampling schedule across the farm for the study. Five sampling batches were conducted for all water sources. Water samples from the reservoir and shrimp ponds were collected on the days of water exchange for the shrimp ponds. The samples from settlement ponds, SF, and HRAP were taken 3 days thereafter

Water sources	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Reservoir	10/4 am	17/4 am	24/4 am	03/5 am	10/5 am
Shrimp pond	10/4 am	17/4 am	24/4 am	03/5 am	10/5 am
Settlement pond	13/4 am	20/4 am	27/4 am	06/5 am	13/5 am
SF	13/4 am	20/4 am	27/4 am	06/5 am	13/5 am
HRAP	13/4 pm	20/4 pm	27/4 pm	06/5 am	13/5 am

Table 2. Analysing methods, practical quantification limits (PQL) and units of nitrogen components included in the research

Parameters	Analysing methods	PQL	Units
Total nitrogen - TN	APHA 4500 N C	0.1	mg/L as N
Total dissolved nitrogen - TDN	APHA 4500 N C	0.1	mg/L as N
Dissolved organic nitrogen - DON	TDN - DIN	0.1	mg/L as N
Dissolved inorganic nitrogen - DIN	DIN = TAN + NO_x	0.1	mg/L as N
Total ammonia nitrogen - TAN	USEPA 103,104,129	0.02	mg/L as N
Oxidised nitrogen - NO_x	APHA 4500 NO_3 I	0.01	mg/L as N
Nitrite - N	TM_NO_2	0.015	mg/L as N

2.4. Data analysis

For the nitrogen water samples, the mean (n = 5) values of each water quality variable were plotted for each of the five water sources. One-way ANOVA and Tukey's post-hoc comparisons were used to compare the differences in the nitrogen content among the water sources. The data analysis presented the key nitrogen variables of total nitrogen (TN), total ammonia nitrogen (TAN), dissolved organic nitrogen (DON), total particulate nitrogen (TPN), and oxidized nitrogen (NO_x).

3. RESULTS AND DISCUSSIONS

3.1. Nitrogen waste in shrimp effluent

The effluent from shrimp ponds significantly increased ($P<0.05$) in the content of all nitrogen components (Table 3) except for oxidised nitrogen (NO_x) compounds compared to the reservoir water. The mean total nitogen (TN) concentration increased 27 times from 0.26 to 6.23 mgN/l compared to the reservoir water. Of these, the TAN content was more than 100 times higher than in the reservoir water, rising from 0.02 to 2.63 mgN/l. The dissolved organic nitrogen (DON) level increased from 0.20 to 1.85 mgN/l, which was 9 times higher than in the reservoir water. Total particle nitrogen fraction (TPN) and oxidised nitrogen (NO_x) levels increased from 0.03 to 1.53 mgN/l and from 0.01 to 0.19 mgN/l, respectively, in the shrimp discharge water. During the sampling period, 25% of the nitrogen in the shrimp pond effluent was TPN, 42% was TAN, and 30% was DON. The NO_x component contributed only a small proportion (3%) to the TN (Figure 5).

There was a clear accumulation of waste nitrogen in the effluent from shrimp ponds at the end of production cycle (around 130 - 160 days of culture). In the present study, the TN content in the prawn effluent was in the same range of the farming system in Thailand (6.5mgN/l) (Thakur & Lin, 2003) and higher than reported for several prawn farms (2-3mgN/l) in Australia (Preston *et al.*, 2001; Jackson *et al.*, 2003b). The present results, however, showed that the dissolved nitrogen component (TDN) formed the dominant nitrogen fraction (76%) in the effluent, while Jackson *et al.* (2003b) reported predominantly particulate nitrogen (TPN) in the prawn effluent from other culture systems. The variations in the content and contribution of the different nutrient wastes in the shrimp ponds across the culture systems was not surprising, and was related to the differences in stocking densities and farming management practices, such as feeding strategies, food quality, and water exchange regimes (Thakur & Lin, 2003; Martin *et al.*, 2010). The higher the stocking density used, the higher the amount of waste generated (Martin *et al.*, 1998).

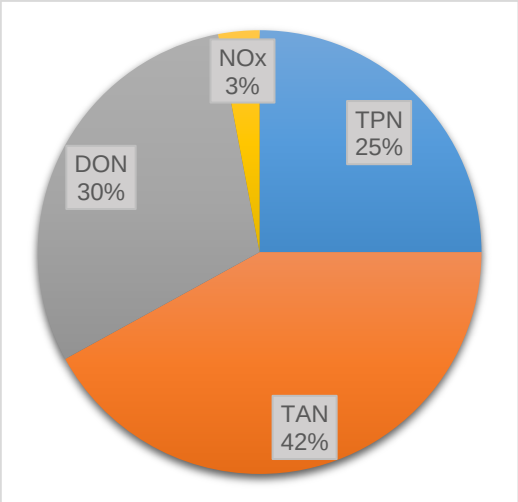

Figure 5. Comparative pie chart of nitrogen components in the wastewater from an intensive prawn farm

Note: The total nitrogen (TN) was comprised of total particulate nitrogen (TPN), total ammonia nitrogen (TAN), dissolved organic nitrogen (DON), and oxidised nitrogen (NO_x)

Table 3. Changes in the total nitrogen (TN) load and the main nitrogen components, including total ammonia nitrogen (TAN), total dissolved organic nitrogen (DON), total particulate nitrogen (TPN), and dissolved nitrogen (NO_x), and total phosphorous (TP) of the water samples after each treatment step

Nutrients	Reservoir	Prawn discharge	Sedimentation	Sand-filter	HRAP
TN (mg l^{-1})	0.25 ± 0.067^{a}	6.23 ± 0.471^{b}	3.30 ± 0.306^{ab}	2.40 ± 0.203^{a}	1.33 ± 0.203^{a}
TAN (mg l^{-1})	0.02 ± 0.003^{a}	2.64 ± 0.202^{b}	1.82 ± 0.442^{ab}	0.74 ± 0.186^{a}	0.17 ± 0.094^{a}
DON (mg l^{-1})	0.20 ± 0.089^{a}	1.85 ± 0.146^{b}	0.62 ± 0.111^{a}	0.69 ± 0.124^{a}	0.64 ± 0.124^{a}
TPN (mg l^{-1})	0.03 ± 0.010^{a}	1.53 ± 0.030^{b}	0.73 ± 0.090^{a}	0.33 ± 0.088^{a}	0.21 ± 0.046^{a}
NO_x (mg l^{-1})	0.01 ± 0.003^{a}	0.19 ± 0.031^{ab}	0.14 ± 0.066^{ab}	0.65 ± 0.097^{b}	.31 ± 0.079^{ab}

Note: Data are shown as mean ± SE for each variable. The values with different letters (a or b) in the same row indicate significant differences (Tukey's HSD, P<0.05)

Regarding the specific nitrogen fractions, present results showed the dominance of nitrogen (TAN) among the TDN forms contributing 55% of the TN in the shrimp pond effluent. A number of previous studies also reported the dominance of TAN in shrimp farming systems (Fvnge-Smith & Briggs, 1994) while others reported the major contribution of DON (Jackson *et al.*, 2003b; Molnar *et al.*, 2013). In a culture system, TAN is produced by microbial remineralization processes within the sediment and by direct excretion by prawns (Burford &Williams, 2001; Burford *et al.*, 2003), while the high level of DON has been suggested to be as a result of leaching from commercial feed (Burford & Williams, 2001). A very low concentration of oxidised nitrogen (NO_x) in the wastewater corroborated with the previous findings in prawn farming systems (Jackson *et al.*, 2003b; Thakur & Lin, 2003; Molnar *et al.*, 2013). The low concentrations of NO_x was a result of the low nitrification rate, which is one of the major limitation of intensive farming systems as it is difficult to design prawn ponds to maximize natural microbial processes (Hargreaves, 1998). In these situations, the nitrification process of oxidizing ammonia to nitrate is usually limited by aerobic

heterotrophs and other chemoautotrophic bacteria, which have higher affinities for oxygen, outcompeting nitrifying bacteria for oxygen (Nizzoli *et al.*, 2006). Variations in the contribution of different nitrogen forms, particulate versus dissolved, and organic versus inorganic, highlight the importance of having multiple steps in the treatment process to treat aquaculture wastewater effectively.

3.2. Treatment of sedimentation pond

The sedimentation pond significantly reduced TN concentration compared to the shrimp effluent water, with significant reductions in total particulate nitrogen (TPN) and dissolved organic nitrogen (DON) (Fig. 8 & 10; $P<0.05$). The sedimentation decreased TN content by 47% from 6.2 to 3.3 mgN/l on average. The main effects were reductions in TPN from 1.5 to 0.7 mgN/l, and DON from 1.85 to 0.62 mgN/l. In addition, the sedimentation step also reduced the TAN content by 31% from 2.6 to 1.8 mgN/l although the NO_x concentration of the sedimentation water was essentially the same as the shrimp discharge water. The nitrogen composition of the sedimentation water was 55% TAN, 22% TPN, 19% DON, and 4% NO_x (Fig. 6, 7, 8, 9, & 10).

The present results confirm the effectiveness of sedimentation ponds in reducing the particulate nitrogen load (TPN). The TPN removal efficiencies by sedimentation (52% reduction) was within the range of that reported for other shrimp farming systems (<60%) in Thailand and Australia (Preston *et al.*, 2001; Jackson *et al.*, 2003a). According to Castine *et al.* (2013), the particulate fractions which settle in sedimentation ponds due to gravitation range in size from 1 to 100 μm. However, the initial reduction of particulate material in the sedimentation pond eventually leads to the mineralization of organic nitrogen and the release of dissolved nitrogen into the water column from the settled particles (Jackson *et al.*, 2003a) due to the activities of microbial communities (Castine *et al.*, 2012). Therefore, the levels of TAN and DON are generally increased in sedimentation water over time (Preston *et al.*, 2001; Jackson *et al.*, 2003a; Erler *et al.*, 2007). A number of factors may influence the removal effectiveness of sedimentation ponds, including effluent composition, residence time, pond design, and pond management (Preston *et al.*, 2001). These could be the causes of the wide fluctuations in the contents of nitrogen components of sedimentation water samples as shown in the present study. In fact, the sedimentation ponds need to frequently removed sediments to improve their removal effectiveness.

3.3. Treatment by sand filter and *Ulva* seaweed

Sand filter and HRAP further decreased the total nitrogen (TN) concentration by 32% from 3.3 to 1.33 mgN/l, of which sand-filter contributed 15% and HRAP contributed 16% to the reduction. The main removal efficiency of sand-filtration reduced 41% of the TAN by converting the nitrogen into oxidised nitrogen (NO_x) resulting in an increase in NO_x levels 4.5 times from 0.14 to 0.65 mgN/l (due to nitrification). The conversion processes are based on the presence of both heterotrophic and nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) in the sand-filter (Prochaska & Zouboulis, 2003). However, nitrifying bacteria are generally dominant in the sand-filter, leading to slower ammonification compared to nitrification (Prochaska & Zouboulis, 2003). In addition, the treatment by sand-filteration also removed 26% of the TPN component in the shrimp effluent.

The HRAP, which was cultivating *Ulva ohnoi,* proved to be effective in the removal of inorganic nitrogen including the reduction of TAN from 0.74 to 0.17 mgN/l, and the reduction of NO_x from 0.65 to 0.31 mgN/l. Noticeably, HRAP showed a high capacity in the removal of NO_2 which is a toxic compound to culture animals, reducing it from 0.46 to 0.20 mgN/l. The DON content, however remained largely unchanged compared to the sedimentation

water. Therefore, after the three treatment steps, the treated water contained 1.33 mgN/l TN with the final relative contribution of 48% DON, 23% NO_x, 16% TPN, and 13% TAN. Compared to the initial shrimp effluent, 79% of the TN content was removed in the final treated water, in which 94% TAN, 86% TPN, and 65% DON was reduced. Compared to the reservoir water, no significant difference in the contents of nitrogen compounds in the treated water was shown despite being five times higher. This could be due to the considerable variation between sampling batches (see standard errors -SE in the Figure 6, 7, 8, 9, &10).

The combination of sand-filteration and *Ulva* seaweed proved to be an effective complementary treatment option for the further reduction of total nitrogen (TN), particularly TAN and TPN in the wastewater. The sand-filter was designed for further removal of all sized nitrogen components, mainly suspended and fine solids to complement the sedimentation pond (Castine *et al.*, 2013). Compared to the sedimentation water, the major changes in the sand-filtered water were the reductions of 47% TAN and 26% TPN, along with the 4.5 times increase in NO_x content (due to nitrification). The high DON concentration in the sand-filtered water could be due to the outcompeting of nitrifying bacteria compared to the heterotrophic bacteria leading to limitations of ammonification as explained above.

The final treatment step by HRAP with *Ulva* seaweed was effective in removing TAN and NO_x, with a higher removal efficiency for TAN. The high removal efficiencies of seaweed, particularly *Ulva,* for dissolved inorganic nitrogen (both TAN and NO_x) have been extensively reported (da Silva Copertino *et al.*, 2009; Khoi & Fotedar, 2011; Aníbal *et al.*, 2014; Rabiei *et al.*, 2014). In this case, the higher removal efficiencies for TAN compared to NO_x may be due to a higher preference of *Ulva* species for ammonia than nitrate (da Silva Copertino *et al.*, 2009). These results highlighted the key role of seaweed and the suitable combination of treatment steps in the purification of nitrogen waste in the effluent from shrimp farms.

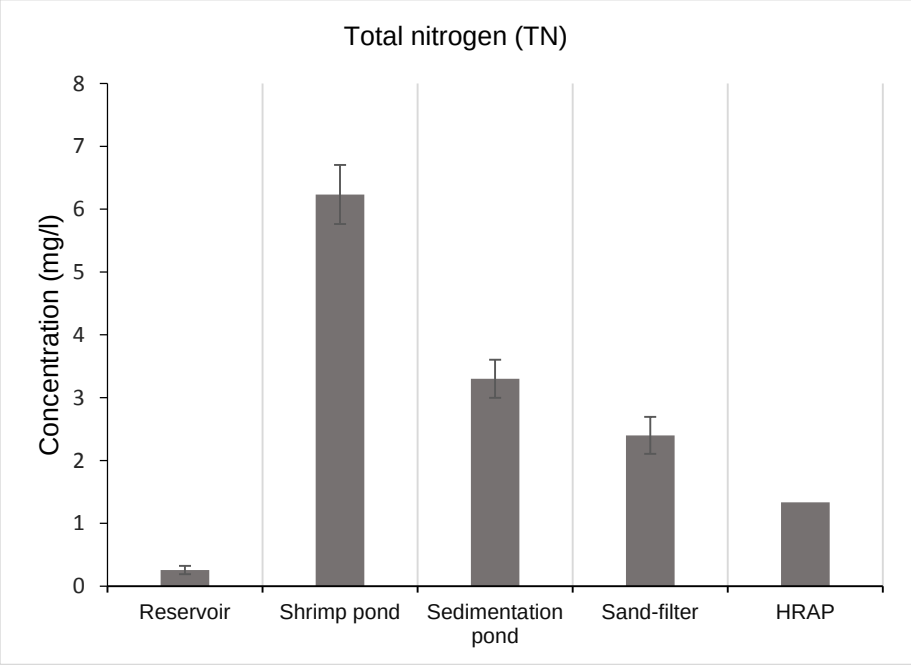

Figure 6. The changes in total nitrogen (TN) concentration in the water samples across the farm.

Note: Data are shown as mean ± SE

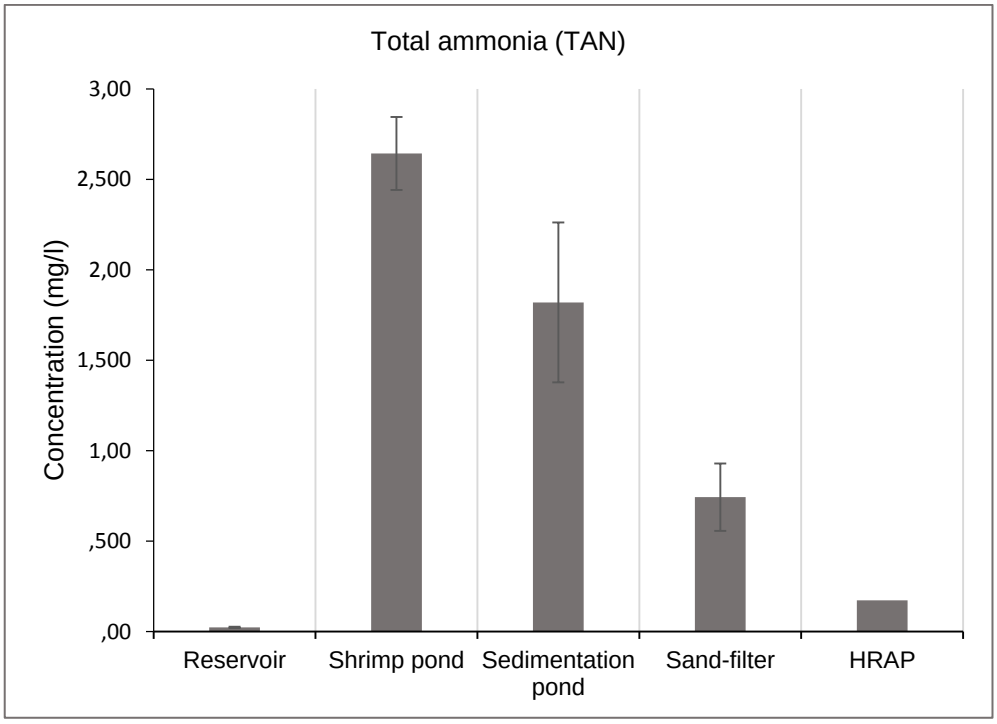

Figure 7. The changes in total ammonia nitrogen (TAN) concentration in the water samples across the farm

Note: Data are shown as mean ± SE

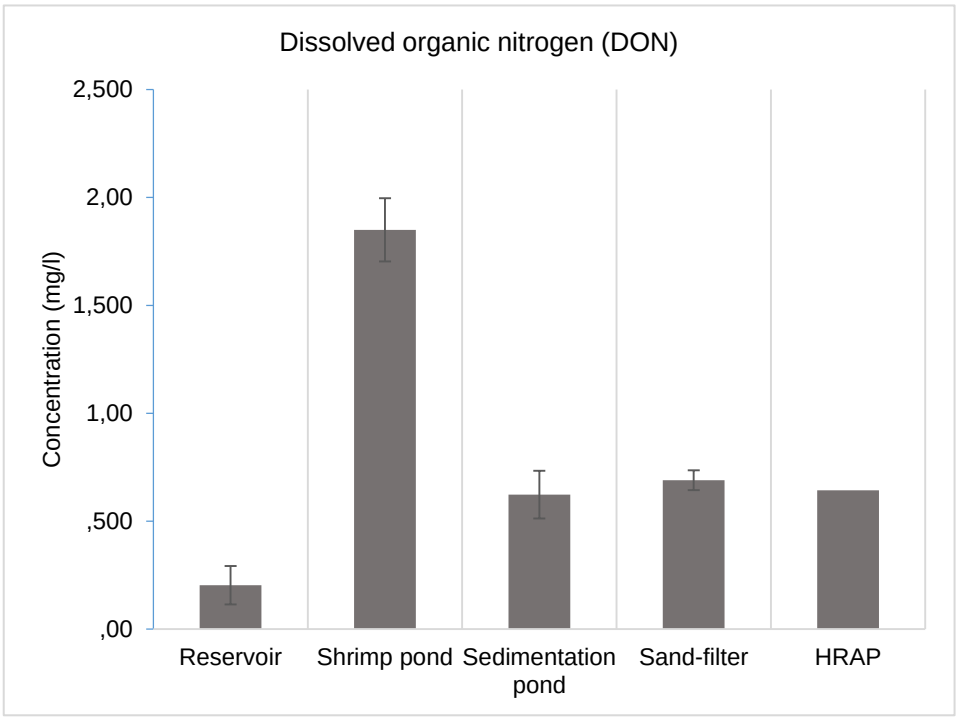

Figure 8. The changes in dissolved organic nitrogen (DON) concentration in the water samples across the farm

Note: Data are shown as mean ± SE

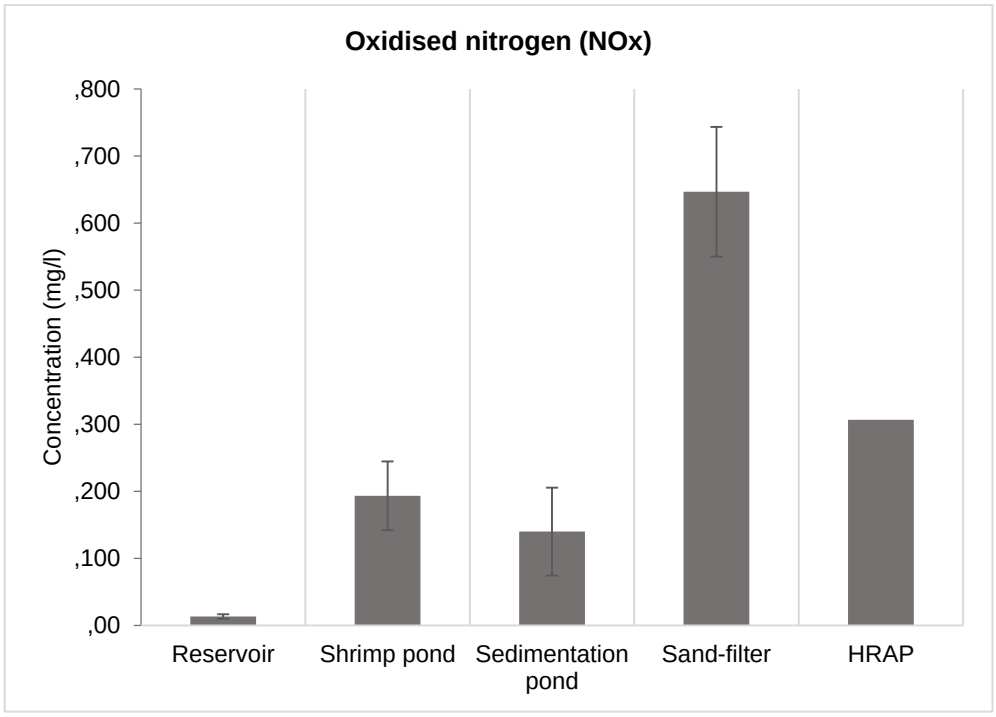

Figure 9. The changes in the oxidized nitrogen (NO_x) concentration in the water samples across the farm

Note: Data are shown as mean ± SE

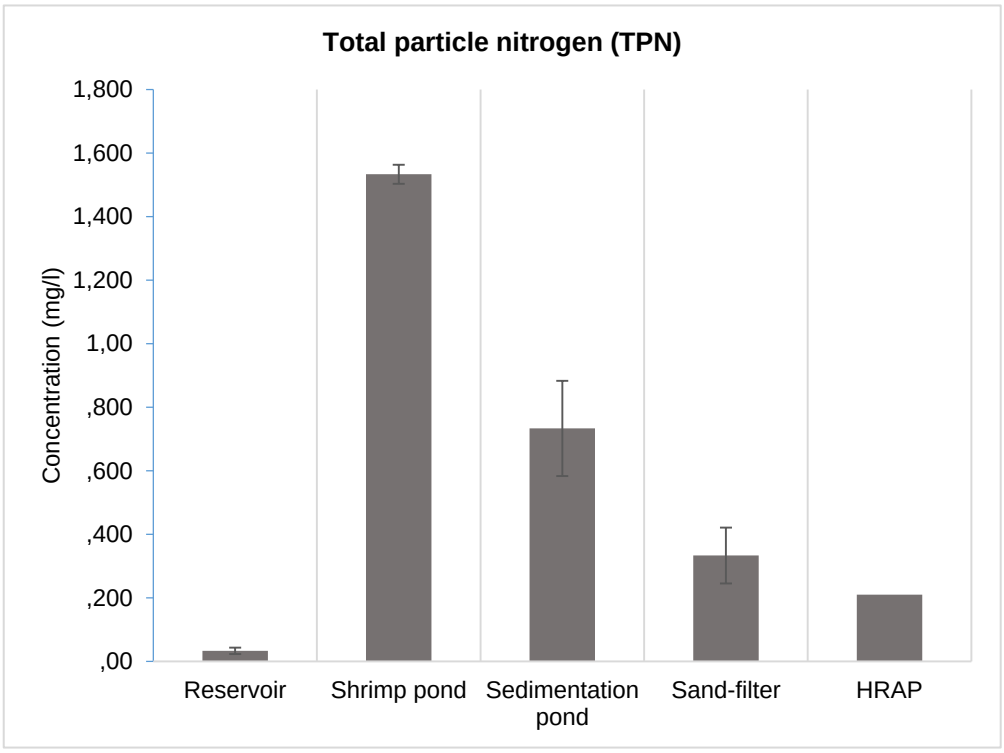

Figure 10. The changes in the total particle nitrogen (TPN) concentration in the water samples across the farm

Note: Data are shown as mean ± SE values for the content of each water source

4. CONCLUSION

The effluent from intensive shrimp ponds accumulated high levels of most nitrogen compounds, particularly total ammonia nitrogen (TAN) and dissolved organic nitrogen (DON). A treatment system consisting of seaweed purification was highly efficient in the removal of nitrogen from waste water (reduced TN by nearly 80%). Compared to the reservoir water, the content of waste nutrients in the new treated water showed no significant differences. However, the TN content in the new treated water was 5-times higher than that in the reservoir water indicating room for improvement. To improve this, the operation of treatment systems should be upgraded in order to reduce the DON component by converting it into inorganic nitrogen forms, and reduce the flowing rate through the sand-filter and the final HRAP step to allow the effective removal of dissolved inorganic compounds.

Acknowledgement

This study was part of the Bioremediation project under support of MBD Energy Limited Company and Pacific Fisheries company in Australia. We thank staff of the Macro group at James Cook University and staff of the Pacific Reef Prawn farm, especially Dr. Nicholas Paul, for their support to the study.

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ACUTE HEPATOPANCREATIC NECROSIS DISEASE: A NEW EMERGING THREAT IN THE SHRIMP INDUSTRY - A REVIEW

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Received date: 02.03.2017 Accepted date: 24.05.2017

ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) is considered to be a new emerging shrimp disease currently affecting brackish water shrimp aquaculture in Southeast Asia including Viet Nam. It was first recorded in shrimp farms in China in 2009 and then in Vietnam in 2010, Malaysia in 2011, Thailand in 2012, and Mexico in 2013. It has been found that AHPND is caused by unique bacterial strains belonging to the *Vibrio* group. AHPND-causing strains have been found to be carried in a plasmid harboring virulent Pir A/B genes that code for the two toxin proteins inducing AHPND in shrimp. This paper will update all research progress on AHPND, including epidemiology and risk factors, etiology, disease diagnosis, disease prevention, and management. This review will serve as a useful introduction for researchers who are currently unfamiliar with AHPND and will hopefully encourage readers to participate in the research efforts to reduce AHPND's impact on shrimp aquaculture.

Keywords: Acute hepatopancreatic necrosis disease -AHPND, shrimp, *Vibrio parahaemolyticus*, *Vibrio*.

Bệnh hoại tử gan tụy cấp: Mối đe doạ mới đối với nghề nuôi tôm công nghiệp

TÓM TẮT

Bệnh hoại tử gan tuỵ cấp (AHPND) đã và đang được xem là một bệnh tôm nguy hiểm mới xuất hiện gần đây ảnh hưởng đến nghề nuôi tôm nước lợ ở khu vực Đông Nam Á trong đó có Việt Nam. Bệnh được ghi nhận đầu tiên tại các trang trại nuôi tôm ở Trung Quốc vào năm 2009, sau đó lần lượt được phát hiện thấy ở Việt Nam và Malaysia năm 2011, Thái Lan năm 2012 và Mexico năm 2013. Các nhà khoa học đã phát hiện ra rằng AHPND bị gây ra bởi các chủng vi khuẩn thuộc nhóm *Vibrio*. Các chủng vi khuẩn gây bệnh AHPND có chứa Plasmid mang các gen độc lực Pir A/B mã hoá cho các protein gây ra hiện tượng hoại tử cấp ở gan tuỵ tôm. Bài viết này sẽ cập nhật tất cả các thông tin về AHPND, bao gồm các thông tin về dịch tễ học và các yếu tố nguy cơ, tác nhân gây bệnh, chẩn đoán bệnh, và biện pháp phòng trị bệnh. Đây sẽ là nguồn thông tin hữu ích cho các nhà nghiên cứu quan tâm đến AHPND cũng như khuyến khích các nhà khoa học tiếp tục nghiên cứu để góp phần ngăn ngừa, giảm thiểu ảnh hưởng của AHPND đối với nghề nuôi tôm nước lợ.

Từ khoá: AHPND, Bệnh hoại tử gan tuỵ cấp, tôm, *Vibrio, Vibrio parahaemolyticus.*

1. INTRODUCTION

According to FAO estimates, to feed the world in 2050, agricultural output must increase by over 70 percent (www.fao.org). Among these agriculture outputs, aquaculture production has taken over as a major supply factor. Aquaculture, especially shrimp culture, makes valuable contributions to the local, national, and regional economies through goods and services sold in domestic and export markets.

As a result of the rapid changes of shrimp culture worldwide in terms of increasing both farming areas and culture densities, newly emerged diseases and occurrences of other diseases have increased year to year. The shrimp farming industry has been suffering from many serious infectious diseases such as

white spot syndrome virus (WSSV), yellow head virus (YHV), Taura syndrome virus (TSV), etc. Since 2009, and a new emerging disease known as Early Mortality Syndrome (EMS), descriptively called as Acute Hepatopancreatic Necrosis Disease (AHPND), has been a major issue of concern for economic losses in the shrimp farming industry (Leano and Mohan, 2012; FAO, 2013).

The aim of this paper is to review the current information on AHPND, including epidemiology, etiology, as well as disease diagnosis, and control measures in hatcheries and shrimp farms.

2. EPIDEMIOLOGY AND RISK FACTORS

2.1. Geographical distribution of AHPND

AHPND caused mass mortality in China (2009) initially, and then in Vietnam (2010), followed by Malaysia (2011), Thailand (2012) (FAO, 2013), and Mexico (2013) (Fig. 1) (De Schryver *et al.,* 2014; Zorriehzahra and Banaederakhshan, 2015). Recently, the presence of AHPND has also been confirmed in the Philippines in 2015 (de la Pena *et al.,* 2015; Dabu *et al.*, 2017), Latin America in 2016 (Han, 2017), and Korea in 2016 (according to information from National Institute of Fisheries Science in Korea, unpublished data).

In China, shrimp farming in Hainan, Guangdong, Fujian, and Guangxi provinces suffered during the first half of 2011 with almost 80% losses. In Malaysia, the outbreaks of AHPND resulted in a significant drop in *P. vannamei* production, from 70,000 million tonnes in 2010 to 40,000 million tonnes in 2011 (Leano and Mohan, 2012). In Thailand, related to the impact of AHPND, shrimp production dropped from an all-time high of approximately 600,000 metric tons in 2011 to less than 200,000 in 2014 (Thitamadee *et al.*, 2016). In Mexico, the disease caused about 118 million USD economic losses in part of this country (De schryver *et al.*, 2014).

In Vietnam, AHPND affected almost all shrimp production areas throughout the country with a total affected shrimp area of around 28,000 hectares in the year 2012. According to DAH (Department of Animal Health) reports, although the shrimp culture areas affected by AHPND decreased significantly from 2012 to 2016, the affected culture locations increased from 192 communes to 299 communes, respectively (DAH reports, 2014; 2015; 2016; Fig. 2).

Fig. 1. Geographical distribution of AHPND

Source: Zorriehzahra and Banaederakhshan, 2015

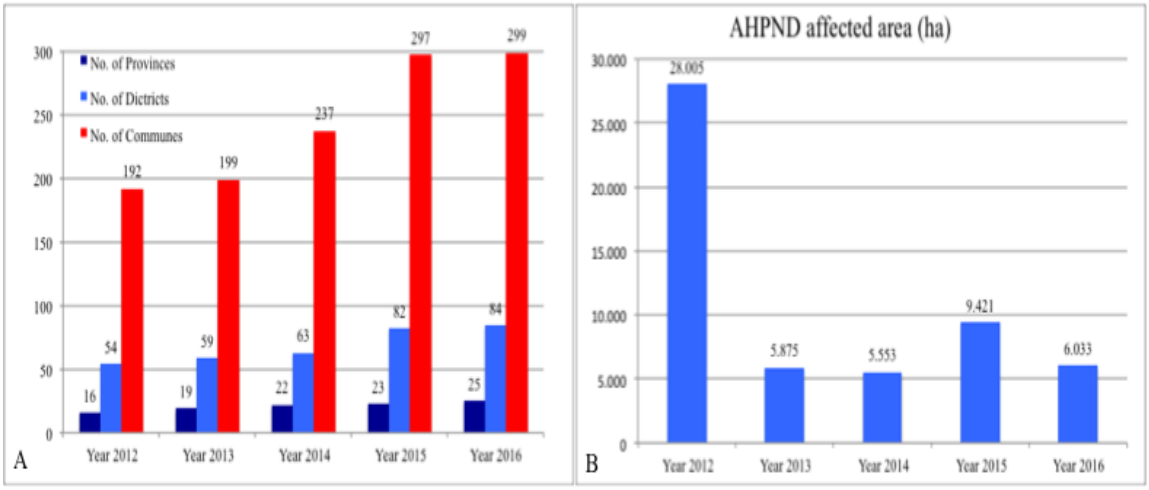

Fig. 2. Spread of AHPND in Vietnam throughout years

Note: A. Affected location; B. Affected area (ha)
Source: DAH reports 2014; 2015; 2016,

2.2. Susceptible species

Important cultivated shrimp, including black tiger shrimp (*Penaeus monodon*) and white leg shrimp (*P. vannamei*), have been reported to be susceptible to AHPND. The disease has also been reported in *P. chinensis* in Southeast Asia while other shrimp species are resistant or less susceptible to AHPND (OIE, 2013). The main susceptibility stage of shrimp is during the early cultivation period approximately 35 days after stocking (Hong *et al.*, 2016).

2.3. Transmission mode

Based on experimental and natural observations, AHPND could be transmitted by oral routes and cohabitation (OIE, 2013). Affected water, affected cultured shrimp, affected PLs, and broodstocks have been considered as materials transferring AHPND pathogens.

It is important to consider that AHPND is caused by unique bacterium strains belonging to the *Vibrio* group and carries virulence genes. *Vibrio* has been consistently identified as one of the dominant bacteria in the natural intestinal flora of penaeid shrimp and is widespread throughout the shrimp aquaculture industry. Moreover, it is known that the virulence genes causing AHPND are located on the plasmid and thus are easily spread between *Vibrio* strains via horizontal mode because the plasmids can transfer genetic material from bacteria to bacteria, leading to a virulence switch and the production of toxins (Corteel, 2016). Taken together, it seems that the spread of pathogens causing AHPND is quite easy in shrimp ponds.

2.4. Factors contributing to the spread of AHPND

According to FAO (2013), Zorriehzahra and Banaederakhshan (2015), Corteel (2016), and Nguyen *et al.* (2016), the following conditions have been considered as risk factors contributing to the incidence and prevalence of AHPND:

(1) High salinity water (Salinity > 5)

(2) High pH water (pH > 7)

(3) Temperature fluctuations

(4) Low oxygen conditions

(5) High amount of *Vibrio* spp. in the water

(6) Sludge/organic substrate in pond bottoms

(7) High stocking density

(8) Lack of reservoir ponds and water treatment

(9) Pond preparation irregularly

(10) Stress for shrimp

(11) Feed mismanagement

(12) Sensitivity of species

(13) Transfer of virulence genes caused acute necrosis of HP cells between *Vibrio* species

(14) Poor/affected shrimp stock

3. ETIOLOGY

After the first occurrence and spread of AHPND in Southeast Asia, initial studies focused on the recognition of pathogens. Several parameters such as cypermethrin, environmental pollution, parasites, viruses, harmful algae, and probiotics were tested for their critical roles on disease spread (FAO, 2013; Hong *et al.*, 2016). However, in early 2013, the causative agent of AHPND was identified as a unique strain of *Vibrio parahaemolyticus* (Tran *et al.*, 2013).

By using multiple whole genome alignments among AHPND-causing and non-AHPND-causing bacterial strains, the unique AHPND-causing strains (VP_{AHPND}) were found to be carried in a plasmid (pVA1) harboring virulent Pir toxin genes, including PirA and PirB genes (Kondo *et al.*, 2014; Lee *et al.*, 2014; Lightner, 2014; Lo *et al.*, 2014; Tinwongger *et al.*, 2014; Yang *et al.*, 2014; Han, 2016). The Pir A/B toxin genes that code for the two toxin proteins (12.7 kDa and 50.1 kDa) that induce AHPND in shrimp have been reported to be similar to the Pir A/B toxin gene known from *Photorhabdus* spp., members of the family *Enterobacteriaceae*. The plasmid pVA1 also carries a cluster of genes related to conjugative transfer, hence, this plasmid may potentially be able to transfer not only among *V. parahaemolyticus* strains but also to different bacterial species (Dong *et al.*, 2017). Fortunately, VP_{AHPND} isolates characterized so far pose no threat to human health (FAO, 2016).

Afterward, several studies presented that another species of *Vibrio*, non-*V. parahaemolyticus*, can also cause AHPND in shrimp (LinThong *et al.*, 2014; Dang *et al.*, 2016). Recently, a *V. harveyi* strain was also identified as a causative agent of AHPND in shrimp cultured in Vietnam (Kondo *et al.*, 2015) along with *V. sinaloensis* (85 % homology) isolated from East Malaysia shrimps (LinThong *et al.*, 2014) and *V. campbellii* isolated from China (Dong *et al.*, 2017) and Latin American shrimp farms (Han, 2017). All of these strains were identified to harbor Pir genes and cause AHPND in shrimp (LinThong *et al.*, 2014; Kondo *et al.*, 2015; Dang *et al.*, 2016; Dong *et al.*, 2017; Han, 2017).

4. DISEASE DIAGNOSIS

4.1. Clinical signs

AHPND caused mass mortality (up to 100%) in shrimp at 20-45 days after stocking of post-larvae. The most important clinical symptoms consist of lethargy, corkscrew swimming, and pale coloration as well as empty or interrupted gut. Infected shrimp constantly reveal an abnormal hepatopancreas (HP) such as shrunken or swollen and discolored (Fig. 3) (Leano and Mohan, 2012; OIE, 2013; Dang *et al.*, 2016). Specific clinical signs such as soft and dark shell, wasting, anorexia, and discoloration of the HP are the major clinical signs of AHPND (Zorriehzahra and Banaederakhshan, 2015).

4.2. Histopathology

Hepatopancreas (HP) is the main infected organ in this disease. The major lesions should be observed in the HP during acute progressive degeneration with initial decreases of R, B, and F-cells followed by an obvious reduction of mitotic activity in E-cells. The development of lesions is noticed from proximal to distal with dysfunction of R, B, F, and finally E-cells, with affected HP tubule mucosal cells presenting prominent enlarged nuclei, rounding, and sloughing into the HP tubules (Fig. 4) (Leano and Mohan, 2012; Lightner *et al.*, 2012).

4.3. Molecular Biotechnology

With the purpose of controling of AHPND, researchers have attempted to develop methods for early diagnosis of this disease, eventually leading to the construction of PCR and LAMP kits for rapid, sensitive, and inexpensive detection of the disease from several research groups.

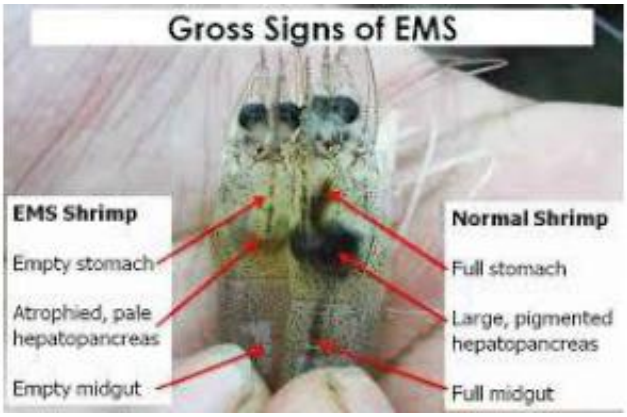

Fig. 3. Gross signs of *P. vannamei* affected by AHPND in Vietnam

Source: DV Lightner, 2012

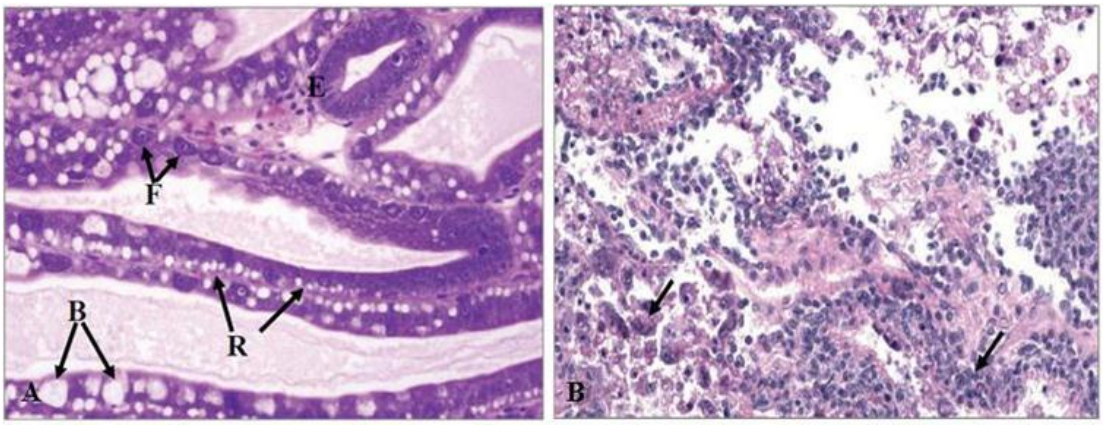

Fig. 4. AHPND-affected HP at acute phase showing acute sloughing of tubular epithelial cells and haemocyte infiltration (Fig. 4B), compared with normal HP with intact tubules and distinct F, B, and R cells (Fig. 4A)

Source: FAO, 2013

Firstly, a PCR test kit was developed by Taiwan and Thailand research groups for AHPND diagnosis based on 2 primer sets called AP1 and AP2 (Shrimp News, 2014). After that, a new and improved PCR method (called AHPND detection version 3) using AP3 replaced the AP1 and AP2 method and a two-tube nested PCR (called AHPND detection version 4) using AP4 was developed for AHPND detection (Sirikharin *et al.*, 2014; Dangtip *et al.*, 2015). The sequences of AP1, AP2, AP3, and AP4 primers are given in Table 1. Also, a set of primers applying toxin Pir genes for detection of AHPND was designed by a Japanese research group (Tinwongger *et al.*, 2014; Table 1).

Recently, a duplex PCR assay that serves to diagnose AHPND and, further, to distinguish among pathogenic AHPND strains collected from various geographic regions was developed by an American research group. For this method, two pairs of primers, MX-345F/R (or Asia-382F/R) and VpPirA-284F/R, are added to a single tube during PCR (Han, 2016). The VpPirA-284F and VpPirA-284R primers (Table 1) allow this test to indicate the presence of the toxin gene located in the AHPND pathogenic bacteria while the MX-345F/R or Asia-382F/R allow the test to distinguish among types of AHPND bacterial strains.

Table 1. Information of primers used for detection of AHPND

Name of primers	Primer sequence	PCR product (bp)	Reference/Source
AP1	F: CCTTGGGTGTGCTTAGAGGATG R: GCAAACTATCGCGCAGAACACC	700	Flegel and Lo (2014)
AP2	F: TCACCCGAATGCTCGCTTGTGG R: CGTCGCTACTGTCTAGCTGAAG	700	Flegel and Lo (2014)
AP3	F: ATGAGTAACAATATAAAACATGAAAC R: GTGGTAATAGATTGTACAGAA	336	Sirikharin *et al.* (2014)
AP4.1	F: ATGAGTAACAATATAAAACATGAAAC R: ACGATTTCGACGTTCCCCAA	1269	Dangtip *et al.* (2015)
Ap4.2	F: TTGAGAATACGGGACGTGGG R: GTTAGTCATGTGAGCACCTTC	230	
TUMSAT-Vp3	F: GTGTTGCATAATTTTGTGCA R: TTGTACAGAAACCACGACT	360	Tinwongger *et al.* (2014)
Toxin	F: GTGGAAATGGTGAACTTGCG R: TACGAGCATTGTTAGGGGTTA	630	Japanese researchers
VpPirA-284	F: TGACTATTCTCACGATTGGACTG R: CACGACTAGCGCCATTGTTA	284	Han (2016)

In addition, a commercial diagnosis test kit called IQ Plus™ EMS/AHPND kit (GeneReach Biotechnology Corp) was designed to use in disease diagnosis. A loop-mediated isothermal amplification (LAMP) assay combined with colorimetric nanogold (AuNP) was developed for detection of AHPND disease with a total assay time of approximately 50 minutes. This LAMP assay was 100-times more sensitive than the 1-step PCR detection method (Suebsing *et al.*, 2014).

To date, all the kits/methods mentioned-above can be used to screen for AHPND bacteria in environmental samples, broodstock feeds, feces from broodstock, post larvae before stocking shrimp ponds, and suspect shrimp under cultivation to help to reduce the probability of AHPND outbreaks.

5. DISEASE PREVENTION AND MANAGEMENT

Because AHPND is caused by a bacterium, not a virus, the use of effective antibiotics against bacteria are available but have some limitations such as antibiotic resistance and public health problems. On the other hand, antibiotic usage also kills the useful bacteria in the pond that can increase disease potency. Therefore, disease prevention and health management are the best ways for disease control.

Based on the current knowledge of AHPND transmission, the contributions of risk factors to the spread of the disease and the common use of *Vibrio* in shrimp aquaculture systems, a holistic management approach is really necessary in order to successfully minimize the damage bacteria inflict on cultured shrimp. According to Han (2016), a holistic approach to AHPND management is mentioned as follows: Firstly, basic good practices have to be established in the management of shrimp culture systems to provide an optimal and stable environment. Secondly, the shrimp's health can be reinforced by optimizing nutrition and supportive supplements for the immune system. Thirdly, the presence of virulent bacteria will be reduced and opportunistic bacteria will be prevented from getting a chance to overwhelm the shrimp.

5.1. Notes for shrimp hatcheries (Zorriehzahra and Banaederakhshan, 2015; Corteel, 2016; Hong *et al.*, 2016)

Use specific pathogen free (SPF) broodtocks.

Disinfect nauplii and materials used in the hatcheries as well as improve the formulated diets for broodstocks to eliminate the risk of pathogen transfer via natural feeds.

Pay attention to the health, water, and feed management for high quality and healthy shrimp post larvae production as the first way to disease control.

Check post larvae health and test responsible organs for the presence of *Vibrio* and other organisms, and ensure their quality control.

Continually control different parts of the hatchery and shrimp larvae for the detection of *Vibrio* bacteria, especially *V. parahaemolyticus*, in order to decrease the bacterial load and prevent disease.

5.2. Notes for shrimp farms (Zorriehzahra and Banaederakhshan, 2015; Corteel, 2016; Hong *et al.*, 2016)

Prepare ponds carefully;

Stock healthy and high quality post larvae; avoid a high stocking density;

Use water storage ponds (reservoirs);

Decrease or control pH; reduce and keep water salinity low;

Use high quality and safe food and immune stimulant material for improving shrimp immunity;

Monitor total *Vibrio* during culture period;

Utilize the Biofloc techniques to significantly increase the survival rate of infected farms;

Use a poly culture system (shrimp and tilapia or marine fish) to prevent disease and/or increase the survival rate in affected systems for the reasons as follows:

+ Zooplanktons use from the bacteria as feed and decrease the bacterial load in the water and fish feed on the zooplanktons decrease the bacterial total count indirectly;

+ The blue-green algae population decreases due to fish feed and after that decrease, decreases the bacterial population;

+ The antibacterial effects of fish mucosa decrease the density of bacteria in the ponds.

6. FUTURE PERSPECTIVES

AHPND has been a serious challenge for the shrimp farming industry, not only in Viet Nam, but also worldwide, because the causative agents (*V. parahaemolyticus* and non-*V. parahaemolyticus*) are common inhabitants of coastal and estuarine environments all over the world and are often found naturally associated with shrimp aquaculture systems. The transfer of plasmids carrying toxin genes between bacteria is facilitated by the aquatic environment. Therefore, innovative farm management and appropriate biosecurity are necessary to alleviate the AHPND crisis to ensure sustainable shrimp production.

Applying disinfectant during pond preparation will reduce the risk of horizontal transfer. Management of sludge on pond bottoms is another important strategy, since organic matter that accumulates on pond bottoms can also serve as a substrate for *Vibrio* spp., including *V. parahaemolyticus*.

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Vietnam J. Agri. Sci. 2017, Vol. 15, No. 9: 1158 - 1169 Tạp chí Khoa học Nông nghiệp Việt Nam 2017, 15(9): 1158 - 1169
www.vnua.edu.vn

COMPARATIVE SMALL INTESTINAL HISTOMORPHOMETRY OF MUONG INDIGENOUS PIGS AND VIETNAMESE WILD BOARS

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Received date: 31.07.2017 Accepted date: 25.10.2017

ABSTRACT

The development of histomorphology structures of small intestine in pigs is affected by food consumptions. In the present study, twelve Muong indigenous pigs and nine Vietnamese wild boars were investigated to compare the morphology of small intestine. The tunica muscularis in duodenum of Muong indigenous pigs was significantly thicker than that in wild boars. In jejunum, the difference was observed in submucosa layer and the crypt depth. In the segment of ileum, the thickness of submucosa and inner mucosa layers of Muong indigenous pigs was significantly greater than that of the wild counterparts. Conversely, the villous height and the cript depth in the wild boars showed higher value. The ratio of villous height/crypt depth in duodenum and jejunum of Muong pigs was higher than that in wild boars but the contrary result was found in the ileum. The distribution of Goblet cells in duodenum of Muong indigenous pigs was 2.8 times more than that of Vietnamese wild boars. The results suggested that the difference in nutrient sources might be a factor to decipher the histomorphology structures of small intestine of pigs.

Keywords: Histomorphology, Muong indigenous pigs, small intestine, Vietnamese wild boars.

So sánh cấu trúc vi thể ruột non giữa lợn Mường và lợn rừng Việt Nam

TÓM TẮT

Sự phát triển cấu trúc vi thể ruột non của lợn chịu ảnh hưởng bởi thức ăn tiêu thụ. Chúng tôi tiến hành thí nghiệm trên 12 lợn Mường và 9 lợn rừng Việt Nam nhằm so sánh cấu trúc vi thể của ruột non. Lớp áo cơ đoạn tá tràng ruột non của lợn Mường dày hơn so với của lợn rừng. Ở đoạn không (hổng) tràng, sự khác biệt có ý nghĩa ($p<0.05$) được tìm thấy ở lớp tổ chức liên kết hạ niêm mạc và độ sâu của ống tuyến ruột. Tại hồi tràng, lớp hạ niêm mạc và niêm mạc của lợn Mường dày hơn so với ở lợn rừng, chiều cao lông nhung và độ sâu của ống tuyến ruột ở lợn rừng có giá trị cao hơn ($p < 0.05$). Tỷ lệ chiều cao lông nhung/độ sâu ống ruột tại tá tràng và không tràng của lợn Mường cao hơn so với tỷ lệ này của lợn rừng. Tuy nhiên, ở hồi tràng, tỷ lệ này ở lợn rừng lại đạt cao hơn. Số lượng tế bào Goblet tại tá tràng ruột lợn mường cao hơn 2.8 lần so với ở lợn rừng. Kết quả cho thấy sự khác biệt trong nguồn dinh dưỡng có thể là môt yếu tố quyết định sự khác biệt trong cấu trúc vi thể ruột non của hai nhóm lợn trong nghiên cứu này..

Từ khoá: Cấu trúc vi thể, lợn Mường, lợn rừng Việt Nam, ruột non.

1. INTRODUCTION

Small intestines are major site for digestion and nutrient absorption which are specially designed to accommodate enlarged surface areas (Herdt. 2007). Contributing to the digestive and absorptive abilities of small intestines are the architectural structures of mucosa including the large folds known as *plicae circulares*, fingerlike epithelial projection known as *villi* and brushlike surface membrane covering the villi known as *brush border* (Skrzypek *et al.,* 2007; Biagi *et al.,* 2007).

The digestive ability of small intestines is greatly influenced by quality of diet (Vicente *et al.,* 2009). Although the number and size of villi

are operated by wild genotype (Skryper *et al.,* 2007). Mitchaothai *et al.* (2010) have proven the effect of diet with high calcium on the height of villus. However, it is obviously that the quantity of feed compositions of the wild animal does not reflect individual preferred as well as special species' requirement but only the availability of food supply.

By comparing the morphology of small intestines, not only the individual response reflecting the level of adaptation can be defined but also the preferred feed profile for further conservation of Vietnamese indigenous and wild boars could be partially predicted.

2. MATERIALS AND METHODS

Twelve Muong indigenous pigs (7 M and 5 F; BW, 10.65±2.3kg; body length, BL, 58.83±9.25cms) have been randomly collected from Muong ethnic households in Hoabinh - a northwest mountainous province of Vietnam. Nine Vietnamese wild boars (7 M and 2 F; BW, 13.63 ± 0.57kgs; BL, 70.28 ± 5.84cms) were procured in Langson in far northern of Vietnam. Average age was estimated of 437.83 ± 102.62d for Muong indigenous pigs but not available for Vietnamese wild boars (Biodata of animals were shown in appendix 1). Small intestine samples were taken immediately after death and separately cut from stomach and large intestine.

All the contents were gently removed under tap water. Samples were selected (3x4 cm - longitudinal) from 3 segments including Duodenum, Jejunum and Ileum; and fixed in 10% buffered formalin for further histomorphological studies.

Tissue selected from fixed area was trimmed (≤ 3mm thick) and refixed in neutral buffered formalin up to 48 hours before processing. Samples were processed 18 hours then embedded in melted paraffin.

Paraffin blocks were sectioned in 3µm using microtome then floated on 37°C water bath. The most intact sections were chosen and placed onto the surface of clean glass slides. The slides were left on warming block (up to 4 hours) for drying and the wax starting to melt.

Different segments of the small intestine including duodenum, jejunum and ileum from each individual sample were sectioned into 3 µm for H&E staining and 5 µm for Alcian blue staining. A slight modification of the Orcein-Alcian blue staining method described by Singh and Gorton (1989) was employed for differentiation of sulphated mucins and sialomucins.

Tissue sections were examined at the X5 and X20 magnifications for histometry and goblet cells enumeration under a light microscope (Leica, Japan).

Each sample was measured in triplicates for muscularis externa, submucosa, inner mucosa, villous height and crypt depth. Goblet cells were counted on 20 different fields on each duodenum slide that has been stained with Alcian blue.

Diagram for measurement of studied parameters is described below.

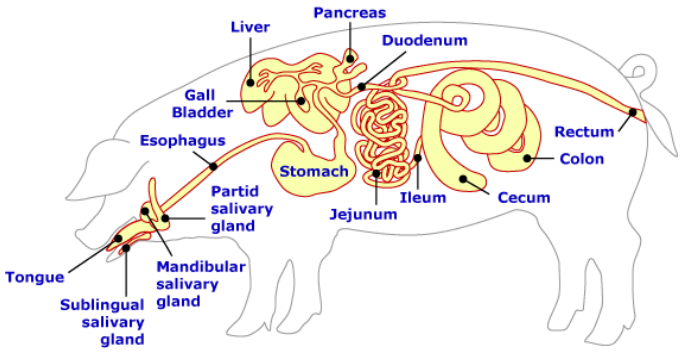
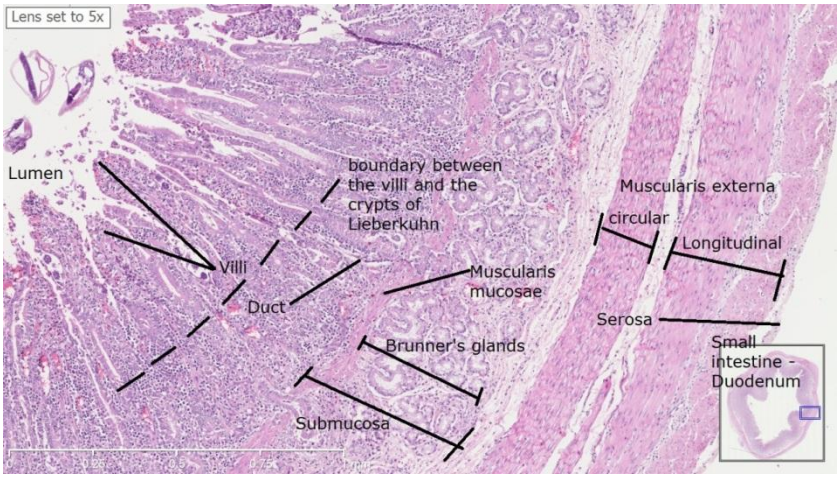


Diagram for small intestinal necropsies

Source:https://courses.ecampus.oregonstate.edu/ans312/ten/swine_1.htm



Source: http://www.onlineveterinaryanatomy.net/content/equine-duodenum-histology-0

Table 1. Comparative histometry of the duodenum (µm; Mean ± S.E.M)

	Muong indigenous pigs	Vietnamese wild boars
Parameter	n = 12	n = 9
Muscularis externa	408.58 ± 22[a]	372.83 ± 9[b]
Submucosa	164.61 ± 15	164.42 ± 7
Inner mucosa	687.84 ± 19	697.28 ± 9
Villus height	349.99 ± 14	353.41 ± 14
Crypts depth	249.25 ± 7	262.95 ± 9

Note: [a,b] Values within row with different superscripts differ at p<0.05

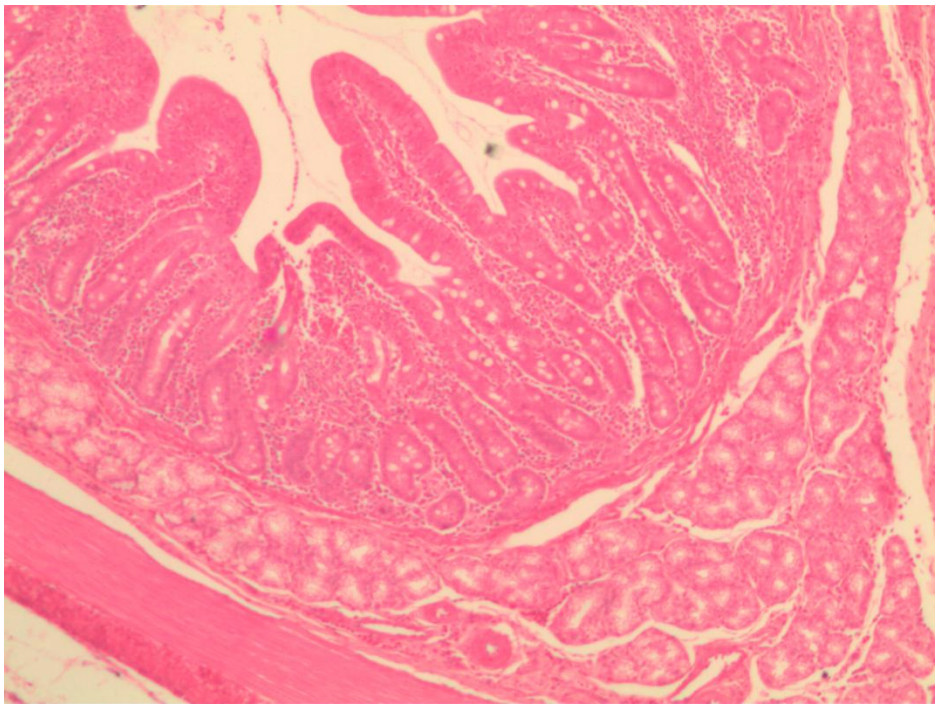


Figure 1. Photomicrograph of the duodenum of Vietnamese wild pig (H&E, X20)

One way ANOVA has been employed for statistical analysis and only data with *p-value* less than 0.05 was considered significant.

3. RESULTS

3.1. Duodenal histometry

The results of morphology measurement of duodenum are presented in Table 1. Two studied subjects revealed similarities of duodenal structure with four out of five measured elements being comparable ($p \geq 0.05$). In this segment of small intestines, only the thickness of tunica muscularis of Muong pigs was significantly ($p < 0.05$) greater than that of the Vietnamese wild boars (408.58 ± 22 vs. 372.83 ± 9).

3.2. Jejunal histometry

The results of morphology measurement of the middle segment of the jejunum are presented in Table 2. In this portion, the submucosa layer of the Muong pig was significantly ($p < 0.05$) thicker than that in the wild pig counterpart. On the other hand, the crypts in the Vietnamese wild boars revealed significantly greater depth than those of the Muong pigs (227 µm vs. 214 µm).

Although the layers of tunica muscular and mucosa of the Vietnamese wild boars were slightly greater than that of the Muong pig, the difference was not significant ($p \geq 0.05$).

3.3. Ileal histometry

The mean values of ileal morphometry are given in Table 3. Among the three structural layers of ileum, the Muong pigs presented an elevation of the thickness of both submucosa and tunica mucosa compared to that of the Vietnamese wild boars ($p < 0.05$). Conversely, the height of ileal villi and the cript depth of Vietnamese wild boars ileum were significantly ($p < 0.05$) greater than that of Muong pigs (291.29 ± 14 vs. 266 ± 21 and 204.46 ± 6 vs. 191.17 ± 12, respectively).

Table 2. Comparative histometry of the jejunum (µm; Mean ± S.E.M)

	Muong indigenous pigs	Vietnamese wild boars
Parameter	n = 12	n = 9
Muscularis externa	411.41 ± 46	450.73 ± 35
Submucosa	338.29 ± 71[a]	188.46 ± 9[b]
Inner mucosa	630.15 ± 26	637.81 ± 28
Villus height	288.23 ± 25	282.92 ± 18
Crypts depth	213.75 ± 13[a]	226.99 ± 8[b]

Note: [a,b]Values within a row with different superscripts differ at p<0.05

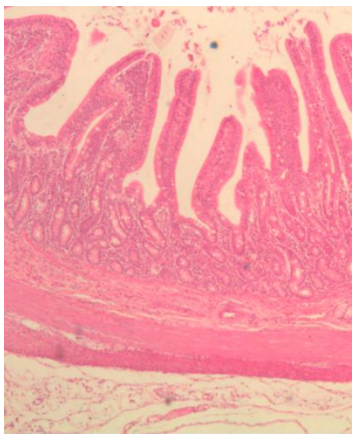


Figure 2. Photomicrograph of the Jejunum of Muong indigenous pig (H&E, X10)

Table 3. Comparative histometry of the ileum (µm; Mean ± S.E.M)

	Muong indigenous pigs	Vietnamese wild boars
Parameter	n = 12	n = 9
Muscularis externa	450.31 ± 50	446.92 ± 25
Submucosa	505.68 ± 57[a]	429.79 ± 33[b]
Inner mucosa	616.27 ± 32[a]	585.05 ± 21[b]
Villus height	266.30 ± 21[a]	291.29 ± 14[b]
Crypts depth	191.17 ± 12[a]	204.46 ± 6[b]

Note: [a,b]Means (± S.E.M) within a row with different superscripts indicate significant differences at p<0.05

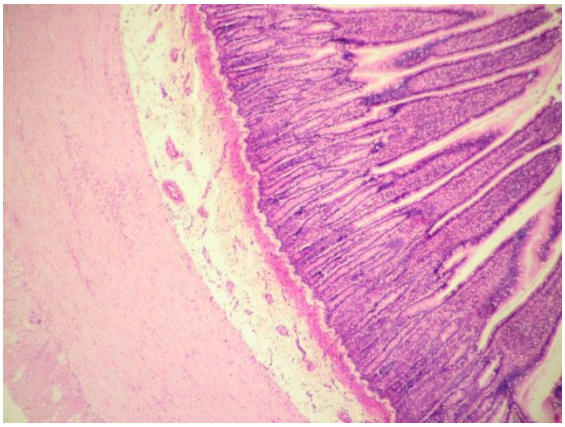


Figure 3. Photomicrograph of the Ileum of Vietnamese wild pig (H&E, 10X)

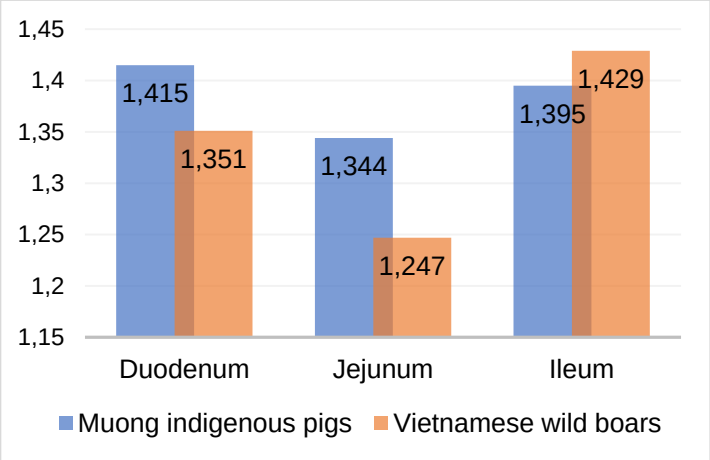


Figure 4. Comparative vilus/crypts of small intestine

3.4. Comparative ratio of villous height and crypt depth of small intestines

Figure 4 shows the comparison between the ratio of villus height and crypt depth of the small intestine in the three different regions.

In the two first segments, i.e., the duodenum and jejunum, the villus/crypt ratio of Muong indigenous pigs was higher than that of the Vietnamese wild boars. However, at the ileal end, this ratio was greater in the Vietnamese wild boars (1.429 ± 0.216 vs. 1.395 ± 0.187).

3.5. Quantities of duodenal goblet cells

The total number of duodenal goblet cells enumerated in both species is shown in Figure

4. The analysis revealed that the number of Goblet cells was significantly ($p<0.05$) higher in the Muong indigenous pig as compared to that of the Vietnamese wild pig (790.56 vs. 274.14).

Figure 6 and Figure 7 show the duodenal histology of the Muong indigenous and Vietnamese wild boars, respectively. It is evident that the goblet cells in the Muong indigenous pig were evenly distributed in all layers (Figure 3). However, fewer goblet cells were seen in the Vietnamese wild boars which also almost completely disappeared on the epithelial layer of villus (Figure 4).

Figures 8 - 10 show the histology of the duodenum of both pig breeds being stained by Orcein and AB to demonstrate the different type of mucus producing cells, i.e., sulphated and carboxylated mucins, respectively. The sulphated and carboxylated mucins are seen as brown to black and light to slight blue colour, respectively.

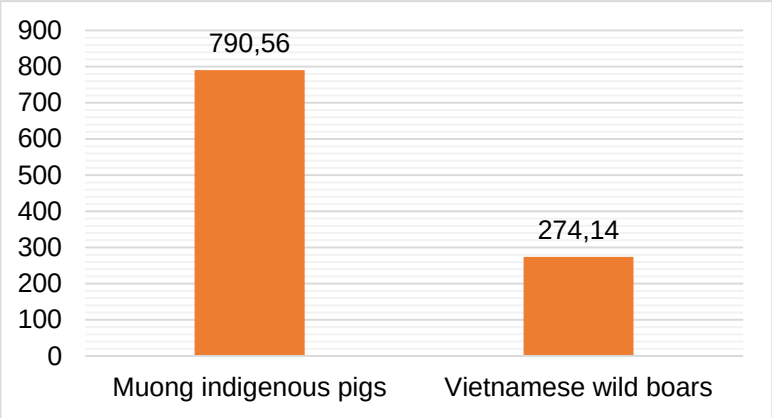


Figure 5. Comparative quantities of duodenal goblet cells

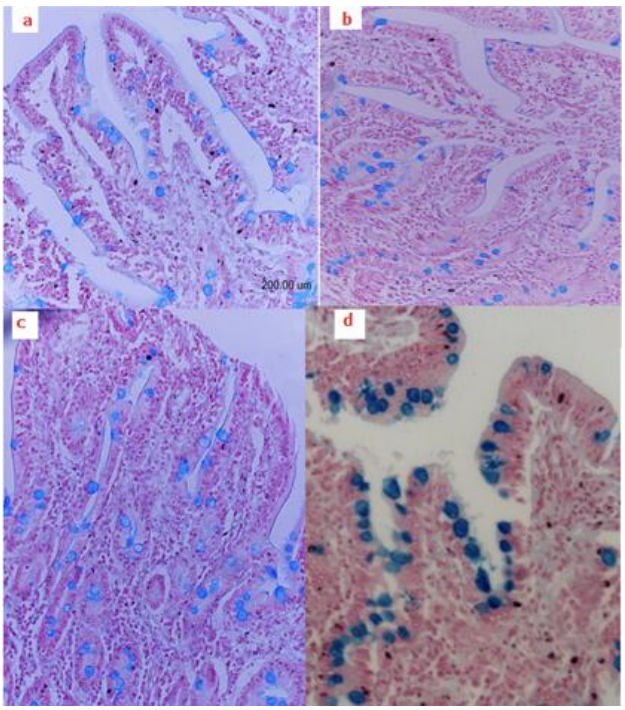


Figure 6. Photomicrograph of the duodenum of the Muong indigenous pig

Note: The goblet cells (blue color) is evident at almost all layers (Alcian blue staining, 400X).

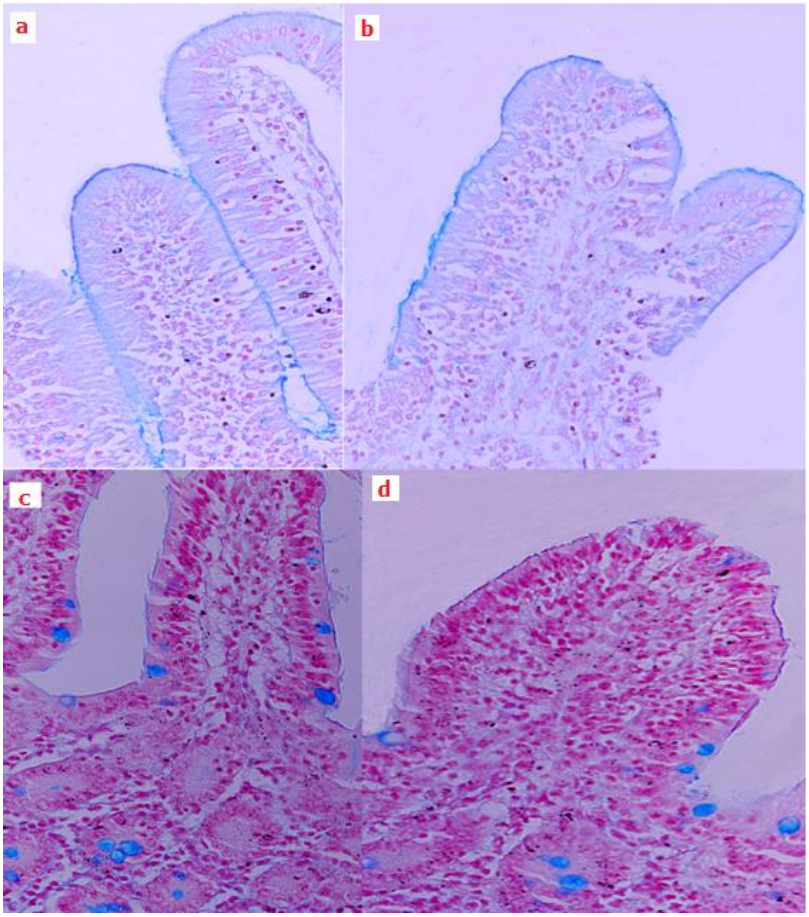


Figure 7. Photomicrograph of goblet cells (blue color) in duodenum of the Vietnamese wild boars

Note: Not only they are scarcely seen but disappear at the epithelial surface (Alcian blue staining, 400X).

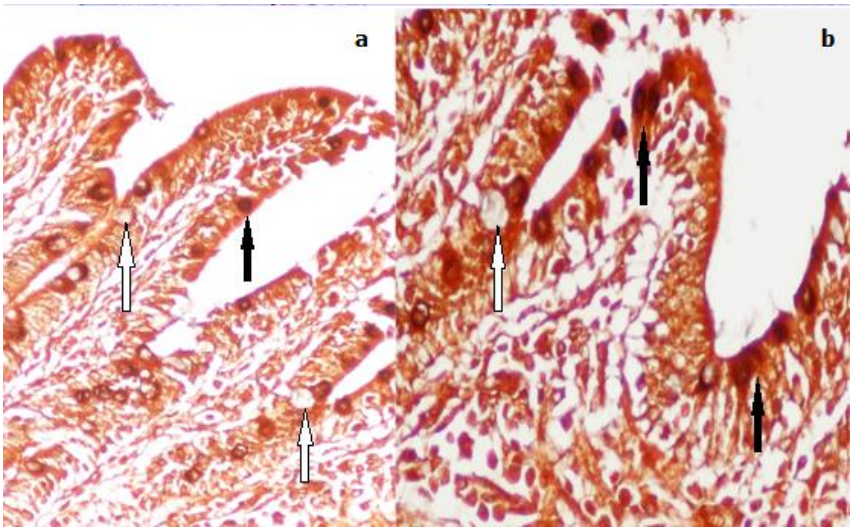


Figure 8. Photomicrograph of the duodenum of the Muong indigenous pigs

Note: It predominantly composes of sulphated mucins (brown to black - black arrow) compared to very few sialomucins (slight blue - white arrow) on epithelium of duodenum (Orcein-AB; a: 200X; b: 400X).

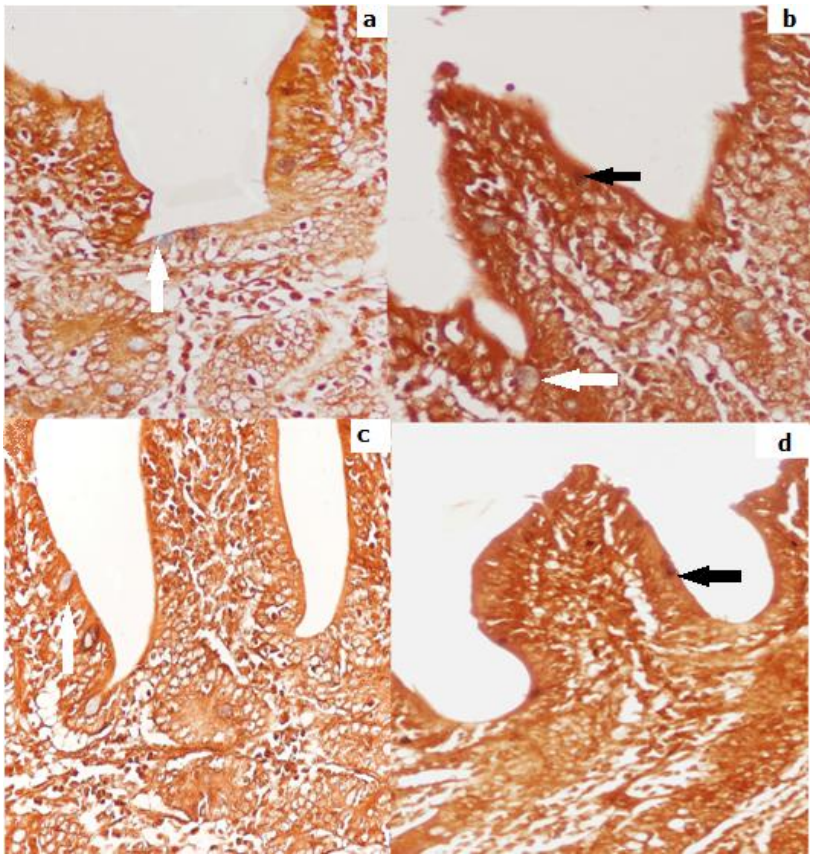


Figure 9. Photomicrograph of the duodenum of the Vietnamese wild boars. Note the scarcity of goblet cells population containing sulphated mucins (brown to black - black arrow) and sialomucins (slight blue - white arrow). (Orcein-AB; a and b: 400×; c and d: 200×)

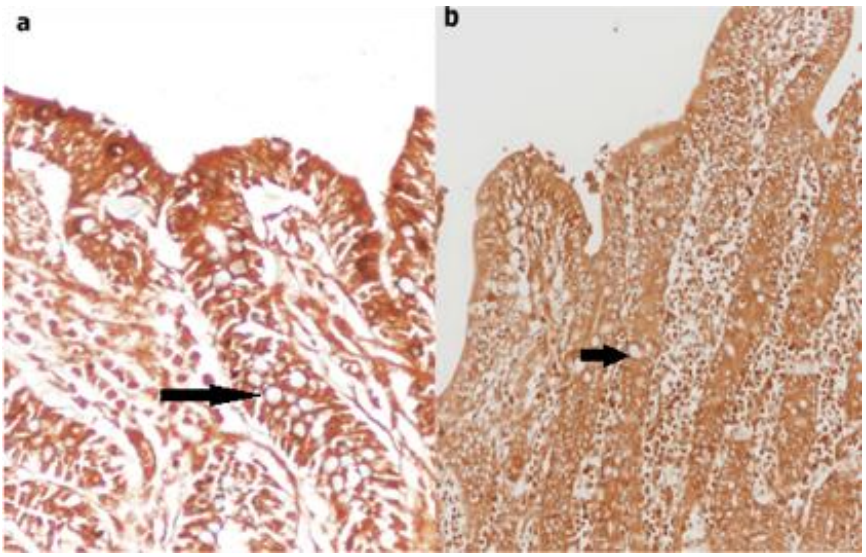


Figure 10. Photomicrograph of the duodenum of the Muong indigenous (a) and Vietnamese wild boars (b). Although it is evident that there is different quality and quantity of goblet cells on the epithelium, the carboxylated mucins producing cells in the crypt are still of majority (light to slight blue - black arrow; Orcein-Alcian blue; a: 400×; b: 200×)

4. DISCUSSION

In general, there exists vast similarity in the morphometry of the duodenum in Vietnamese wild boars and Muong indigenous pigs especially with regards to the thickness of submucosa and tunica mucosa with the exception of a thicker muscularis externa in the Muong indigenous pigs. This discrepancy occurs probably due to physiological activity since it is the main site for digestion, in particular mixing of feed with digestive secretions (Herdt, 2007). With reference to digestive function, Uhr (1995) has proven that the influence of genetic improvement during domestication rather than originated modification led to an increase in small intestinal dimensions. Uhr (1995) stated that in the domestic pig as seen in the Muong indigenous breed in this study, the greater development of tissue at the site where digestion took place resulted from the correlation between high rate of digestion and high protein biosynthesis as compared to that of the Vietnamese wild boars. In the wild boars, the diet is usually those of low digestibility and protein.

The increased thickness of the small intestine of the Muong pigs could have arisen due to the existence of the Peyer's patches. The appearance of Peyer's patches can be considered as genetically fixed in submucosa of digestive tract (Uhr, 1995). Furthermore, it is especially abundant in ileum and is affected by the stimulation of microbes immigrating from colon (Driessen *et al.,* 2002; Fayed *et al.,* 2010). It is likely that the manner of being reared under an "intensive" farming system would have created stress to the pigs. Thus, the gut environment of Muong pigs is supposedly to be more sensitive to disease leading to much more lymphatic tissue (Peyer's patches) to confer a better immune system (Uhr, 1995).

However, this finding is in contrast to those reported elsewhere documenting wild boars to harbor greater number of Peyer's patches (Skrzypek *et al.*, 2007). Nevertheless, in that study the pigs used were crossbred which would have assumed the conformity towards what is seen in the Muong pigs which were reared intensively as opposed to that of the "purebred" Vietnamese wild boars used in this study. Likewise, several findings in this study bear resemblance to those of regulating of shallower of transversal furrows in duodenum, longer of villi in jejunum of Polish landrace/Pietrain in comparison with Duroc/Hampshire/wild boar crossbreed piglets (Skrzypek *et al.*, 2007).

Although GI morphology was greatly influenced by feed composition, frequency of food intake, body shape and size (Kararli, 1995), the ileum still remains conserved as it is the main site for absorption (Gourevitch D. 2005). Therefore, it is not surprising or unexpected for the presence of statistical difference in ileal morphology between the Muong indigenous and Vietnamese wild boars. In this study, due to the continuously changing and different living conditions between both breeds, ileal histomorphometry are highly differentiated (Uhr, 1995).

On the contrary with the middle layer, the tunica mucosa diminished towards the end of the length of small bowel and conformed to the typical morphology of small intestines (Gourevitch, 2005; Budiño *et al.*, 2005). Such trend was also seen in the in rats (Vigueras *et al.*, 1999; Hosoyamada and Sakai, 2005), geriatric dogs (Kuzmuk *et al.*, 2005) and geese (Liu *et al.,* 2010).

In considering the ratio of villus height and crypts depth (VH/CD ratio), the influence of quality and quantity of food intake must be considered. The present study yielded lower ratios than those observed in weaned and monitored feed-intake commercial pig breeds (Tang *et al.,* 1999; Brundige *et al.*, 2008) but higher ratios than in dietary restricted and malnourished pigs (Nunez *et al.*, 1996). This discrepancy could have arisen from the different sampling time and also nature of feeding in the two breeds studied.

Although some authors considered VH:CD ratios as the standard index of absorptive abilities (Pluske *et al.*, 1996; Pluske *et al.*, 1997; Montagne *et al.*, 2003). However, the response of these ratios maybe reversed by a variety of factors. Hyperplasia of crypt taht are not accompanied with the increasing villous height may lead to a

decrease of VH:CD ratio (Kien *et al*., 2007). The influence of microflora and external environment on the enterocyte turnover rate has been proven to result in shorter villi and deeper crypt (Kelly *et al*., 1994).

Conversely, the VH:CD ratio may not be unchanged even though the diminishing factors of the both element are obvious. Fasting condition reduces cell division in the crypts which might led to villous atrophy (Nunez *et al.,* 1996; Fernandez-Estivariz *et al.,* 2003). The high calcium diet may promote the VH:CD ratio in wild boars mainly by diminished depth of crypts but there was no affection of this dietary component on the villous height (Mitchaothai *et al*., 2010). Therefore, the increase/decrease of both villous height and crypt depth influenced by different factors must be sifted when making conclusion of absorptive abilities of small intestines based on VH:CD ratio. Sifting of these factors is almost impossible in this study as its main aim was mainly the histomorphometry and further studies should be carried out to determine the factors governing VH:CD in both breeds.

The total number of duodenal goblet cells in this study did not agree with previous researches. This may due to different methodology since almost researchers used villi as a standard unit (Fernandez-Estivariz *et al*., 2003; Brundige *et al*., 2008) instead of scoring total number of cells on field and also different species did not have the same quantities of Goblet cells on epithelium (Paulini *et al*., 1987; Ito *et al*., 2009).

The influence of fiber diet on mucus production of gastrointestinal tract has been documented. Ito *et al.* (2009) have found the relation of soluble fiber and small intestinal mucins activity where higher molecular weight fiber resulted in the greater number of goblet cells. The similar results were obtained by Satchithanandam *et al.* (1990) when they compared the mucin reactivity to 5% guar gum, 5% citrus fiber and fiber-free control diets. The results indicated that statistically greater reaction has been found in both luminal and tissue level of 5% citrus fiber feeding group.

The modeling study carried out by Paulus *et al.* (1993) stated the same origin stem cell of goblet and columnar cells and the shift to goblet cell took place at specific stage in the development of epithelial columnar cells. Therefore, factors affecting turnover proliferation of small intestines and crypt cell division in particularly such as daily food restriction (Nunez *et al*., 1996; Fernandez-Estivariz *et al.,* 2003), prolonged protein depletion or lower nutrition requirement of protein-energy (Sherman *et al*., 1985) are all responsible for the decrease of the mucin production in small intestines. The Vietnamese wild boars are destined to have lower number of goblet cells as their diet albeit fibrous but likely to be of lower digestibility apart from prolonged protein depletion or low protein diet. However, Muong indigenous pigs owing being managed under a much more intensive and commercial system (better feed quality in terms of fiber and protein) have much higher goblet cell population.

In general, the occupying of sulphomucin containing cells on the villi and the predominant of sialomucin producing cells in both deeper areas of the crypt and Brunner' gland in both breeds were in agreement with the earlier observations (Poddlar and Jacob, 1979; Singh and Gorton, 1989).

5. CONCLUSION

The differences of morphometry of small intestine were mainly observed in jejunum and ileum which were the most influenced by the dietary feed intake and also the modify abilities of the absorptive structure to the hash and continuingly changing of nurture condition.

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Appendix 1. Biodata of animals

No.	Code	Body weight (kg)	Body length (cm)	Sex	Estimated age (day)
1	HB1	9,5	50,6	Female	350
2	HB2	8,0	42,5	Male	330
3	HB3	8,2	43,1	Male	330
4	HB4	8.5	45,2	Female	350
5	HB5	8,0	43,0	Male	330
6	HB6	9,3	49,4	Female	350
7	HB7	14,0	67,2	Female	547
8	HB8	12,5	60,2	Male	530
9	HB9	13,8	66,2	Male	547
10	HB10	12,0	57,6	Male	530
11	HB11	12,5	59,8	Male	530
12	HB12	11,5	61,1	Female	530
13	LS1	12,0	61,4	Male	n.d
14	LS2	13,5	69,2	Female	n.d
15	LS3	12,5	66,4	Male	n.d
16	LS4	14,0	73,2	Male	n.d
17	LS5	14,5	75,8	Male	n.d
18	LS6	13,5	64,6	Male	n.d
19	LS7	14,0	71,7	Female	n.d
20	LS8	13,5	69,7	Male	n.d
21	LS9	15,2	80,5	Male	n.d

Note: HB, LS: Hoa Binh, Lang Son province; n.d: Not detected

EFFECT OF SOYBEAN MEAL REPLACEMENT WITH GUT WEED (*Enteromorpha* sp.) AS A PROTEIN SOURCE IN PRACTICAL DIETS FOR BLACK TIGER SHRIMP (*Penaeus monodon*) POST-LARVAE

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Received date: 09.02.2017 Accepted date: 05.05.2017

ABSTRACT

The study was performed to evaluate the use of gut weed (*Enteromorpha* sp.) as a protein source to substitute soybean meal protein in the diets of post-larval tiger shrimp (*Penaeus monodon*). A diet without gut weed meal, considered as a control, was compared with four experimental diets in which soybean meal protein was replaced by different gut weed protein levels, namely 15, 30, 45 and 60%. All diets were formulated to be equivalent in crude protein (40%) and lipids (7%). The feeding trial was conducted in 100 L plastic tanks filled with water at a salinity of 10 ppt, and provided with continuous aeration. Thirty shrimp post-larvae with a mean initial weight of 0.033g were stocked in each tank and fed the test diets for 45 days. Results showed that survival of the shrimp was not affected by the feeding treatments, and ranged from 84.4 to 88.9%. Overall, the growth rate and feed efficiency of the shrimp fed the gut weed-based diets were comparable to or better than animals fed the control diet. Moreover, the shrimp fed diets containing gut weed meal also showed a better formalin resistance than the ones fed the control diet, although a significant difference was only observed between the 30% replacement treatment and the control group indicating the optimal replacement level for tiger shrimp PL diets.

Keywords: *Enteromorpha* sp., growth, *Penaeus monodon*, Soybean meal, stress resistance.

Ảnh hưởng của việc thay thế protein bột đậu nành bằng protein bột rong bún (*Enteromorpha* Sp.) trong thức ăn cho hậu ấu trùng tôm sú (*Penaeus monodon*)

TÓM TẮT

Nghiên cứu được thực hiện nhằm đánh giá việc sử dụng rong bún (*Enteromorpha* sp.) làm nguồn protein thay thế protein bột đậu nành trong khẩu phần ăn cho hậu ấu trùng tôm sú (*Penaeus monodon*). Thức ăn đối chứng không chứa bột rong bún, được so sánh với 4 thức ăn thí nghiệm, protein bột đậu nành được thay thế bằng protein bột rong bún với các mức khác nhau gồm 15, 30, 45 và 60%. Tất cả thức ăn thí nghiệm có cùng hàm lượng protein thô (40%) và lipid (7%). Thí nghiệm được bố trí trong bể nhựa 100 L ở độ mặn 10‰ và được sục khí liên tục. Khối lượng tôm ban đầu là 0,033g được thả 30 con cho mỗi bể và cho ăn thức ăn thí nghiệm trong 45 ngày. Kết quả cho thấy, tỉ lệ sống của tôm không bị ảnh hưởng bởi các nghiệm thức thức ăn, dao động từ 84,4 đến 88,9%. Nhìn chung, tốc độ tăng trưởng và hiệu quả sử dụng thức ăn của tôm ăn thức ăn có chứa protein rong bún tương đương hoặc tốt hơn so với tôm ăn thức ăn đối chứng.Thêm vào đó, khả năng chịu đựng sốc formol của tôm ở các nghiệm thức có rong bún tốt hơn so với nhóm đối chứng nhưng sự khác biệt có ý nghĩa chỉ được tìm thấy giữa nghiệm thức thay thế 30% protein rong bún và nghiệm thức đối chứng. Điều này được xem là mức thay thế tối ưu cho thức ăn của hậu ấu trùng tôm sú.

Từ khóa: Bột đậu nành, *Enteromorpha* sp., *Penaeus monodon*, tỉ lệ sống, tăng trưởng

1. INTRODUCTION

Aquaculture production is highly dependent on commercial feeds and aquafeeds rely on several common input ingredients such as fishmeal, soybean, corn, fish oil, rice bran and wheat powder, for which they compete in the market place with the animal husbandry sector (FAO, 2013). Currently, their availability is a major concern for their high cost and scarcity of raw materials. Moreover, in shrimp farming, the feed cost is the highest proportion and accounts for more than 50% of the total production costs (Long, 2016). In addition, most feed manufactures are using expensive imported fishmeal and soybean meal as protein sources for aquafeeds resulting in high price. Therefore, assessment of cheaper or more readily available alternative plant protein sources such as seaweed, aquatic plants, or by-products from fisheries may reduce the use of imported ingredients in feeds (Cruz-Suárez *et al.*, 2008; FAO, 2013).

Gut weed (*Enteromorpha* spp.) has a high nutritional value containing 9 - 14% protein, 2 - 3.6% lipids, 32 - 36% ash, and n-3 and n-6 fatty acids at 10.4 and 10.9 g/100 g of total fatty acid, respectively. The protein of this seaweed has a high digestibility up to 98% (Aguilera-Morales *et al.*, 2005). Recent investigations revealed that gut weed belonging to green algae and is distributed abundantly in the extensive shrimp farms and other brackish water bodies of the Mekong delta, Vietnam (ITB-Vietnam, 2011). This indicates that a large quantity of gut weed is available for aquaculture feeds. Moreover, several studies have found that gut weed *Enteromorpha* had the best nutritional and functional properties as an ingredient in shrimp feeds, which improved survival, growth, feed efficiency, and stress resistance of cultured species (Cruz-Suárez *et al.*, 2008; Anh *et al.*, 2014; Mondal *et al.*, 2015).

Black tiger shrimp (*Penaeus monodon*) has a high economic value, and is an important cultured species in the Mekong delta. However, according to the Ministry of Agriculture and Rural Development (2015), shrimp diseases have been observed since 2010, but the most widespread devastation due to early mortality syndrome (EMS) has been reported since 2011 in the Mekong Delta causing serious losses for shrimp farming (Long, 2016). The impact of EMS could be minimized by nursing shrimp post-larvae in tanks for a certain period in order to provide the formulation of well-balanced diets and adequate feeding that are of the utmost importance for successful growth out in ponds. The main objective of the study was to determine the optimal replacement level of soybean protein with gut weed protein in practical diets for the black tiger shrimp *P. monodon* post-larvae.

2. MATERIAL AND METHODS

2.1. Experimental diets

Gut weed (GW) were collected from an abandoned intensive shrimp pond, Bac Lieu province, cleaned under tap water, and shade-dried in thin layers for 3 days. The dried GW was then ground into a powder. Soybean meal and fishmeal were supplied by CATACO Company; other ingredients such as squid oil, gelatin, cassava powder, and rice bran were purchased from commercial suppliers. The dietary ingredients were analyzed for chemical composition (Table 1) prior to the formulation of the diets.

Table 1. Approximate composition (% dry matter) of ingredient

Ingredient	Moisture	Protein	Lipid	Ash	Fiber	NFE
Fish meal	11.08	58.14	9.17	21.36	0.56	10.77
Soybean meal	10.43	44.32	2.23	8.25	1.27	43.93
Gut weed powder	6.19	25.44	2.16	24.17	4.14	44.09
Rice bran	9.86	8.52	8.15	21.32	5.33	56.68
Cassava powder	10.87	5.14	1.77	0.69	2.87	89.53

Table 2. Composition of ingredients (% dry matter) and approximate analysis

Ingredients	0% GW	15% GW	30%GW	45% GW	60% GW
Fishmeal	44.50	44.50	44.50	44.50	44.50
Soybean meal	29.19	24.82	20.44	16.07	11.66
Gut weed powder	-	7.63	15.23	22.88	30.49
Rice bran	3.80	8.18	8.76	6.49	4.25
Cassava powder	16.85	9.51	5.75	4.67	4.10
Squid oil	1.16	1.32	0.82	0.89	0.50
Lecithin	0.50	0.50	0.50	0.50	0.50
Premix -Vitamin	2.00	2.00	2.00	2.00	2.00
Gelatin	2.00	2.00	2.00	2.00	2.00
Total	100	100	100	100	100
Approximate analysis of experimental feed					
Moisture	10.16	10.68	10.82	10.45	11.02
Protein	40.68	40.06	39.98	39.82	39.88
Lipid	6.98	6.79	6.65	6.72	6.64
Ash	14.28	16.28	16.34	17.26	18.66
Fiber	2.92	3.21	3.85	4.41	5.77
NFE	35.14	33.66	33.18	31.79	29.05
Ca	2.17	2.26	2.59	2.47	2.54
P	1.32	1.26	1.15	1.08	0.92

Five experimental diets were formulated by replacing 0%, 15%, 30%, 45%, and 60% of the soybean (SB) protein in a practical diet with gut weed protein (Table 2). The 0% gut weed treatment was considered as a control. All test diets were formulated to be approximately isonitrogenous and isolipidic (40% and 7% dietary protein and lipid, respectively).

The 'SOLVER' program in Microsoft Excel was used to establish the formulated feeds. In this program, the approximate composition of the ingredients and those of the diets were preset, in which the proportion of SB protein substituted by GW protein must be precise. Based on the composition of SB meal and GW meal it was therefore possible that other ingredients (e.g. amount of cassava powder, rice bran and squid oil) varied as well in order to keep the gross composition of the resulting diets as similar as possible. The diets were made into sinking pellets, which were oven-dried at 60°C, ground, sieved to the desired particle sizes of 300, 500, 700, and 1000μm, and stored at 4°C for later use.

Approximate analysis (moisture, crude protein, total lipid, fiber, and ash) of the ingredients and experimental diets were determined according to the standard methods of AOAC (2000). Nitrogen-free extract (NFE) was estimated on a dry weight basis by subtracting the percentages of crude protein, lipids, crude fiber, and ash from 100% (Table 2).

2.2. Experimental design and management

A feeding trial was conducted for 45 days at the College of Aquaculture and Fisheries, Can Tho University. The test was set up as a completely randomized design with 3 replicates per treatment. The plastic 100-L tanks were filled with 80 L of seawater at a salinity of 10 ppt, and each tank was provided with continuous aeration. *Penaeus monodon* post-larvae (PL) from one single batch were purchased from a commercial hatchery in Can

Tho city and reared in $1m^3$ tank for 3 days before the start of the trial. During this period, shrimp PLs were fed a mixture of experimental feed. 30 PLs (mean initial weight of 0.033±0.005 g) were transferred into each tank. Shrimp post-larvae were fed to satiation four times a day at about 6:00, 11:00, 16:00, and 21:00 hours at about 20-30% of their body weight. The amount of feed given was adjusted according to daily observation. The faecal matter was siphoned out every morning before the first feeding and 50% of the tank volume was exchanged every 7 days.

2.3. Data collection

Daily water temperature and pH were recorded at 7:00 and 14:00 h using a thermo-pH meter (YSI 60 Model pH meter, HANNA instruments, Mauritius). The concentration of NH_4/NH_3 (TAN), NO_2, and alkalinity were monitored weekly using test kits (Sera, Germany). The water samples in the culture tanks were collected prior to water exchange.

At the beginning of the experiment, 30 post-larvae were randomly taken from the conditioning tank to be measured for individual weight and total length. Shrimp sampling was conducted every fifteen days during which 10 shrimp were randomly taken from each tank, weighed as a group using an electronic balance, and mean weights were determined. Shrimp were then returned to their original tanks. The amount of feed given was adjusted according to these weight measurements. At the end of the feeding trial, the survival, mean individual weight, and total length of the shrimp were determined. The feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated using the following equations:

FCR = Feed intake (dry weight)/Weight gain (wet weight)

PER = Weight gain/Protein intake

The quality of the experimental shrimp was assessed by studying the resistance of the shrimp to formalin shock following the methods of Samocha *et al.* (1998) and modified by Thanh *et al.* (2002). Ten shrimp from each tank were exposed to a 150-ppm formalin solution in a 10 L-glass bottle for 60 minutes. The same temperature and salinity as in the culture medium was maintained with continuous aeration. Dead shrimp were monitored at 10 minute intervals.

The Cumulative Mortality Index (CMI) was calculated by summing the mortality counts noted at each time interval. CMI= $N_{x1} + N_{x2} + N_{x3} + ... N_{x6}$

Where N is the number of dead individuals at times $x_1, x_2, x_3...x_6$. The higher the numeric value of the index, the less resistant the post-larvae are to stress and vice versa.

2.4. Statistical analysis

Data were analyzed using a one-way ANOVA (SPSS, version 16.0) analysis of variance to find the overall effect of the treatment. Duncan's Multiple Range test was used to identify significant differences among the dietary treatment means at a significance level of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Water quality parameters

Table 3 shows that daily mean temperature, pH, and alkalinity fluctuated between 26.2 - 27.8°C, 7.8 - 8.0 and 91- 94 mg $CaCO_3$/L, respectively. Generally, temperature, pH, and alkalinity in each experiment were not much diffent among feeding treatments. The average concentrations of TAN and NO_2^- were not affected by the feeding treatments, varying in the ranges of 0.56 - 0.58 and 0.52 - 0.55 mg/L, respectively (Table 3).

According to Pushparajan and Soundarapandian (2010), maintenance of good water quality in the culture pond is essential for the optimum growth and survival of the black tiger shrimp, *P. monodon*. The levels of physical, chemical, and biological parameters control the quality of pond waters. These authors suggested that the optimum range of temperatures was between 27°C to 30°C, and pH should be maintained from 7.5 to 8.8.

Alkalinity is the buffering capacity of the pond water, and the higher the alkalinity, the better the stabilization of the culture pond is. For successful culture of *P. monodon*, alkalinity is recommended to be in the range of 90-126 mg$CaCO_3$/L (Mohanty *et al.*, 2014).

Whetstone (2002) reported that the toxicity of ammonia and nitrite for shrimp is greatly dependent on environmental factors such as pH, dissolved oxygen, salinity, and temperature. For aquaculture purposes, these factors play an important role in the development, growth, and survival of species exposed to ammonia and nitrite. Chen and Lei (1990) reported that the acceptable concentration for juvenile *P. monodon* (0.27 g) was 3.7 mg/L total ammonia-N and 3.8 mg/L nitrite-N in water of 20 ppt.

From the cited published papers above, water quality parameters in the present study were within acceptable ranges for *P. monodon* growth. Therefore, feeding treatments could be the main factor affecting the performances of experimental shrimp.

3.2. Survival, growth rates, and feed efficiency of *P. monodon* post-larvae fed different test diets for 45 days

The effects of replacing soybean meal protein with gut weed *Enteromorpha* protein in the experimental diets on survival, growth performances, and feed efficiency of *P. monodon* are presented in Table 4.

The results showed that the survival of shrimp feeding on the different test diets for 45 days varied from 84.4% to 88.9%. There were no significant differences (P>0.05) among feeding treatments. This indicated that using gut weed to replace soybean meal protein in the tiger shrimp diet did not affect their survival.

With regards to growth rate, the mean initial weight of shrimp post-larvae was 0.033 ± 0.005 g. After 45 days of the feeding trial, final weight and the specific growth rate (SGR) of experimental shrimp ranged from 0.94 to 0.99 g and 7.34-7.53 %/day, respectively, of which the 15% GW and 30% GW treatments had significantly higher values ($p<0.05$) than those of the control and other substitution treatments. However, for the daily weight gain (DWG) of shrimp, only the 60% GW treatment (0.019 g/day) showed significantly poorer growth ($p < 0.05$) than the remaining feeding treatments (0.020-0.021 g/day).

The average feed intake of shrimp was not statistical different among feeding treatments (P>0.05), ranging from 26.36 to 26.99 mg/shrimp/day. The feed conversion ratio (FCR) was between 1.22 and 1.36, and tended to increase with increasing levels of gut weed protein in the test diets. Statistical results indicated that the 15% GW and 30% GW treatments had significantly lower values (P<0.05) compared to the control and other feeding treatments.

The protein efficiency ratio (PER) in all feeding treatments was in the range of 1.85-2.04, and showed the opposite trend compared with FCR in which the 15% GW and 30% GW treatments were significantly higher than those in the control and other treatments. Nonetheless, there were not significant differences (P>0.05) among the control and the 45% GW and the 60% GW treatments.

Table 3. Water quality in the culture tanks during feeding trial

Treatment	Temperature (°C)		pH		Alkalinity ($mgCaCO_3$/L)	TAN (mg/L)	NO_2^- (mg/L)
	7:00 h	14:00 h	7:00 h	14:00 h			
Control	26.8 ± 0.7	29.4 ± 0.9	7.8 ± 0.4	8.2 ± 0.4	91 ± 12	0.56 ± 0.29	0.53 ± 0.47
15% GW	26.9 ± 0.4	29.5 ± 0.8	7.8 ± 0.4	8.1 ± 0.5	93 ± 11	0.58 ± 0.26	0.52 ± 0.43
30% GW	26.7 ± 0.6	29.8 ± 1.0	7.8 ± 0.3	8.3 ± 0.3	94 ± 10	0.60 ± 0.27	0.53 ± 0.44
45% GW	26.8 ± 0.7	29.6 ± 0.9	7.9 ± 0.8	8.2 ± 0.4	92 ± 11	0.58 ± 0.26	0.55 ± 0.43
60% GW	27.02 ± 0.5	29.5 ± 0.8	7.9 ± 0.4	8.3 ± 0.5	93 ± 12	0.57 ± 0.29	0.54 ± 0.48

Table 4. Survival, growth performance and feed efficiency of *P. monodon* post-larvae over a 45-day feeding trial

Treatment	Control	15% GW	30% GW	45% GW	60% GW
Survival (%)	86.7 ± 5.8^{a}	88.9 ± 1.9^{a}	86.7 ± 1.3^{a}	84.4 ± 5.1^{a}	85.6 ± 3.8^{a}
Initial weight (g)	0.033 ± 0.005	0.033 ± 0.005	0.033 ± 0.005	0.033 ± 0.005	0.033 ± 0.005
Final weight (g)	0.94 ± 0.11^{a}	0.99 ± 0.12^{b}	0.97 ± 0.09^{b}	0.94 ± 0.10^{a}	0.91 ± 0.09^{a}
SGR (%/day)	7.43 ± 0.27^{a}	7.53 ± 0.23^{b}	7.51 ± 0.21^{b}	7.42 ± 0.23^{a}	7.34 ± 0.22^{a}
DWG (g/day)	0.020 ± 0.002^{b}	0.021 ± 0.003^{b}	0.021 ± 0.002^{b}	0.020 ± 0.002^{b}	0.019 ± 0.003^{a}
FI (mg/shrimp/day)	26.46 ± 0.80^{a}	26.99 ± 0.27^{a}	26.26 ± 0.47^{a}	26.69 ± 0.73^{a}	26.36 ± 0.54^{a}
FCR	1.31 ± 0.03^{b}	1.22 ± 0.01^{a}	1.25 ± 0.01^{a}	1.33 ± 0.02^{b}	1.36 ± 0.01^{b}
PER	1.88 ± 0.04^{ab}	2.04 ± 0.02^{c}	1.99 ± 0.01^{c}	1.89 ± 0.02^{b}	1.85 ± 0.02^{a}

Note: Values are mean ± standard deviation. Mean values with different superscripts in the same row are significantly different (P<0.05)

Table 5. Cumulative mortality index (CMI) values measured for *P. monodon* exposed to 250 ppm formalin solution for 60 min

Treatment	Cumulative mortality index
Control	0.83 ± 0.41^{b}
15% GW	0.33 ± 0.52^{ab}
30% GW	0.17 ± 0.41^{a}
45% GW	0.33 ± 0.52^{ab}
60% GW	0.33 ± 0.52^{ab}

Note: Values are mean ± standard deviation. Mean values with different superscripts in the same column are significantly different (P<0.05)

The results in the present study are in agreement with the study of Anh *et al.* (2014) who assessed soybean meal protein substitution with gut weed (*Enteromorpha*) protein or blanket weed (Cladophoraceae) protein at the rates of 20%, 40% and 60% in the practical diets of white leg shrimp (*Litopenaeus vannamei*) post-larvae. The authors found that the survival of shrimp was not affected by the test diets, and ranged from 81.1 to 87.8%. Growth rates of the shrimp fed 20% and 40% replacement levels of gut weed or blanket weed protein in the diets were better or similar to those fed the control diet while at the 60% substitution level, shrimp had poorer growth but there were not any significant differences between the control and the other feeding treatments. Moreover, the feed conversion ratio (FCR) and the protein efficiency ratio (PER) exhibited similar trends as those observed for growth performance, indicating that gut weed and blanket weed could replace up to 40% of soybean meal protein in the diets for *L. vanamei* postlarvae. A similar finding was reported by Cruz-Suarez *et al.* (2006) who compared the potential of *Enteromorpha* meal as an ingredient in shrimp feed formulation with two kelp seaweeds, *Macrocystis* and *Ascophyllum*, by supplementing 3.3% of seaweed in the *Litopenaeus vannamei* diets for 28 days. They found that *L. vannamei* shrimp were larger and had a better feed conversion ratio in the group fed pellets containing *Enteromorpha* than those with *Macrocystis* or *Ascophyllum*.

Another study revealed that red seaweed meal, *Gracilaria* sp., could substitute wheat flour and soybean up to 15% in the shrimp *P.*

monodon diet while 30% dietary inclusion levels resulted in significantly poorer growth and higher FCR compared to the control (0% seaweed) but survival of shrimp was similar among feeding treatments (Briggs and Funge-Smith, 1996). A parallel confirmation was made by Hafezieh *et al.* (2014) who stated that the survival of *L. vannamei* juveniles fed test diets containing different percentages of brown seaweed (*Sargassum illicifolium*) powder from 5 to 15% were similar to those fed the control diet without seaweed meal (95.2-97.0% survival). The specific growth rate of shrimp ranged from 4.68% to 5.68%, and exhibited no significant differences compared to the control diet while the diets at higher inclusion levels (15% and 10%) of brown seaweed exhibited better FCR (1.15 and 1.17) than the 5% and control diets (1.30 and 1.33). Additionally, Felix and Brindo (2014) found that the substitution of fishmeal with raw and fermented *Kappaphycus alvarezii* at four levels, 0 (control), 10%, 20%, and 30%, in the diet for 45 days did not affect the survival of the giant freshwater prawn *Macrobrachium rosenbergii* juveniles (100% survival). Furthermore, 10% raw *K. alvarezii* powder could be incorporated into the prawn's diet without any compromise in growth performance or feed utilization efficiency (FCR and PER) compared with the control diet. However, higher levels of *K. alvarezii* inclusion (20% and 30%) did not perform well. The authors confirmed that the reduced growth of the prawns fed diets containing higher levels of raw seaweed appeared to be due to the increased fiber content due to seaweed in the diets.

3.4. Cumulative mortality index of *P. monodon* post-larvae fed different diets exposed to a 250 ppm formalin solution for 60 min

The cumulative mortality index (CMI) of the shrimp subjected to formalin stress is shown in Table 5. The results indicated that the stress index in the experimental shrimp fed diets containing gut weed protein was lower than in the shrimps receiving the control diet. However, a significant difference was only observed between the 30% GW treatment and the control group.

Moreover, visual observation found that shrimp mortalities in the control diet happened earlier than other test diets. This means that shrimp fed the control diet were less tolerant to formalin stress than in the animals fed gut weed based-diets.

Previous investigations stated that the formalin stress test can be used as a more flexible tool for diagnosing shrimp quality and to formulate appropriate diets (Samocha *et al.*, 1998; Thanh *et al.*, 2002). In the current study, the effect of the dietary treatments on formalin stress resistance displayed a similar pattern as the growth performance, where *P. monodon* shrimp fed based-gut weed feed had formalin stress test results better than animals fed the control diet. Furthermore, a significant difference in CMI was observed between the 30% GW treatment and the control which was in agreement to the investigations that showed that seaweed meal inclusion in aquafeeds at low levels improved growth performance, feed efficiency, and disease resistance of shrimps (Cruz-Suarez *et al.*, 2008). Similar research results were reported by Peixoto *et al.* (2016) who found that European seabass (*Dicentrarchus labrax*) fed feed supplemented either with *Gracilaria* spp., *Ulva* spp., or *Fucus* spp. at 2.5% or 7.5% levels had improved immune and stress responses without compromising growth performance and feed efficiency.

Earlier studies confirmed that gut weed *Enteromorpha* spp. have high nutritional values. For example, gut weed *Enteromorpha linza* was shown to contain 18 amino acids, and high protein and mineral levels, which was higher than that of other seaweeds (*Laminaria japonica*, *Porphyra haitanensis*, *Hizikia fusiformis*, and *Undaria pinnatifida*), and indispensable amino acids were 53% of the total amino acids (Qing *et al.*, 2006). Additionally, gut weed *Enteromorpha* spp. are rich in high unsaturated fatty acids (LN, ARA and EPA) and amino acids, and protein digestibility of gut

weed is high (98%), especially because it contains high levels of astaxanthin (Aguilera-Morales *et al.,* 2005). Similar findings were reported by Anh (2014), gut weed *Enteromorpha intestinalis* collected in the brackish water bodies form Soc Trang and Bac Lieu provinces contained high levels of essential amino acids and was rich in protein and fatty acids which make it a suitable food for fish and shrimp. Besides, Cruz-Suarez *et al.* (2008) reported that diets supplemented with seaweed meal or their extracts, due to the presence of some bioactive compounds (fucoidan, alginates, laminarins, carrageenans, etc.) can enhance immune resistance and improve survival when shrimp are challenged with bacteria or viruses, and can also help the species to combat a stressful environment. Mondal *et al.* (2015) found that green seaweed *Enteromorpha intestinalis* was a natural high content source of astaxanthin (120.78 ppm) and was included in the formulated diets of farmed tiger shrimp (*Penaeus monodon*) in relation to its quality improvement. Astaxanthin showed strong activity as an inhibitor of lipid peroxidation mediated by active forms of oxygen. Among the functions of astaxanthin in aquaculture, the antioxidant properties can be closely associated with stress resistance (Meyers, 1994). Chien *et al.* (2003) reported that *Penaeus monodon* juveniles fed diets supplemented with a 80 mg astaxanthin/kg diet for 8 weeks showed significantly higher resistance to thermal and osmotic stresses than those fed the control diet. The findings cited above can explain the reason why shrimp in the present study fed based-gut weed diets showed better tolerance to formalin shock than those receiving the control diet.

4. CONCLUSIONS

Survival of the experimental shrimp was similar among the feeding treatments, ranging from 84.4% to 88.9%. The dietary replacement of soybean meal protein with 30% gut weed *Enteromorpha* protein seems to be the optimal level for black tiger shrimp *P. monodon* post-larvae as indicated by a significantly higher growth rate, better feed efficiency, and formalin resistance compared to those in the control diet.

Further research should address the *Penaeus monodon* post-larvae response to biotic or abiotic stressors, clarifying the objective role of dietary gut weed *Enteromorpha* protein replacement as immune and antioxidant stimulating.

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IMMUNO-RELATED GENE EXPRESSION OF ANTI-LIPOPOLYSACCHARIDE FACTORS (ALF) AFTER USING IMMUNOSTIMULANT IN KURUMA SHRIMP (*Marsupenaeus japonicus*)

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Received date: 23.02.2017 Accepted date: 29.08.2017

ABSTRACT

Supplementation of immuno-stimulant can act as a potent immune modulator and have the effect on inducion of immune- related genes which involved in host defense. In order to clarify the anti-lipopolysaccharide factors (ALF) expression of Kuruma shrimp (*Marsupenaeus japonicus*) after feeding immuno-stimulant, the supplement containing microalgae and Euglena (Ex400) was selected for the trials. Total RNA was extracted by RNA Iso Plus from the lymphoid organ, intestine and blood of shrimp that was fed with Ex400 diet. Tissues were collected at 0, 3, 7, and 10 days after feeding. ALF transcripts were significantly increased by Ex400 compared to control-diet-fed shrimp (with $P<0.01$) at 3 days post-feeding in the intestine and lymphoid organ. This suggested Ex test diet might stimulate ALF of Kuruma shrimp immune defence in the intestine and lymphoid organ.

Keywords: Anti-lipopolysaccharide factor (ALF), gene expression, immunostimulants, Kuruma shrimp, quantitative real-time PCR.

Biểu hiện của gen ALF liên quan đến miễn dịch sau khi sử dụng chất kích thích miễn dịch trong tôm he Nhật Bản

TÓM TẮT

Chất kích thích miễn dịch là chất giúp tăng cường hoạt động của hàng rào miễn dịch và tác động đến các gen liên quan miễn dịch để bảo vệ của vật chủ. Ex400 được sản xuất từ vi tảo và Euglena là chất kích thích miễn dịch trên tôm được sử dụng để xác định biểu hiện của gen ALF trong tôm he Nhật Bản. ARN tổng số được chiết xuất từ các cơ quan lympho, ruột và máu của tôm tại các thời điểm 0, 3, 7 và 10 ngày sau khi cho tôm ăn Ex400. Trong ruột và cơ quan lympho của tôm, biểu hiện của gen ALF cao hơn so với lô đối chứng tại các thời điểm 3 ngày sau khi cho tôm ăn Ex400 ($P< 0.01$). Kết quả mức độ biểu hiện của gen ALF cho thấy chất kích thích miễn dịch có thể kích hoạt ALF trong hàng rào miễn dịch trong ruột và cơ quan lympho của tôm he Nhật Bản..

Từ khóa: Anti-lipopolysaccharide factor (ALF), biểu hiện gen, chất kích thích miễn dịch, định lượng real-time PCR, tôm Kuruma.

1. INTRODUCTION

Shrimp is considered one of the most internationally important traded fishery commodities in terms of value. The production of cultivated penaeid shrimp species increased exponentially since the early 1970s. However, there is rapid increasing problem with serious disease outbreaks. As shrimps lack an adaptive immune system, they rely on innate immune responses against microbial invasion (Tanekhy & Fall, 2015). A better understanding of the innate immune system of shrimp will undoubtedly help develop strategies in disease control and sustainable shrimp farming.

Anti-microbial proteins (AMPs), the cationic and amphipathic proteins of low molecular weight (<10kDa), play a major role in innate immunity in shrimp lacking adaptive immunity, and studying their functions enriches basic knowledge on immunity and provides possible avenues in formulating disease management strategies in aquaculture (Bachère *et al.*, 2004). AMPs engage mainly to offer an early and first localized line of defense against pathogens (Selsted & Ouellette, 2005). Several AMP families such as penaeidins, crustins, anti-lipopolysaccharide factors (ALFs), histones, and fragments of hemocyanin have so far been described in penaeid shrimps. ALFs are antimicrobial peptides having broad spectra of antimicrobial activity to neutralize gram-negative and gram-positive bacteria, fungi, parasites and viruses (Mekata *et al.*, 2010). ALF, initially isolated and characterized from hemocytes of the horseshoe crab, *Limulus polyphemus (*Miyata *et al.*, 1987), has the endotoxin or lipopolysaccharide (LPS) - mediated coagulation system.

Immuno-stimulants are substances that activate the immune system of animals to make them more resistant to microbial infections (Raa, 1996). The definition has been expanded somewhat to include live organisms or their products that have an impact on the immune system. The use of immuno-stimulants does not generate a specific response to a certain antigen, but causes an overall response that hastens recognition and elimination of a broad range of infectious agents and foreign substances (Sordillo *et al.*, 1997). The present study was carried out to examine the expression of ALF after using immuno-stimulant containing microalgae and Euglena (Ex400) in Kuruma shrimp (*Marsupenaeus japonicus*).

2. MATERIALS AND METHODS

2.1. Animals

Specific pathogen free (SPF) Kuruma shrimps, *Marsupenaeus japonicus,* of 10±0.3g body weight were obtained from Matsumoto Fisheries, Miyazaki, Japan.

2.2. Methods

2.2.1. Experimental design

Prior to feeding experiment, shrimps were acclimatized, reared in aerated seawater tank at 23°C and 30 ppt salinity, and fed with control diet. 3 days. After that, one group of 12 shrimp was fed with Ex400. The second group, used as the control group (fed with control diet fed shrimp, without Ex400). Three shrimps were collected at 0, 3, 7, and 10 days for experiment. The experiment was conducted triplicate. Shrimp body surfaces were washed and disinfected with 70% ethanol, and then the blood, intestine, lymphoid organ were dissected out. One side of lymphoid organ, 200µL of blood, and 1/10 of gut were collected.

2.2.2. RNA extraction

Total RNA was extracted from the sample using RNA Iso plus (TAKARA, Japan). The quantity and quality of all RNA samples were checked using a NanoDrop spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE, USA) at 260nm and 280nm.

2.2.3. Synthesis of cDNA

cDNA was synthesized according to the protocol (TOYOBO, Japan) of ReverTra Ace qPCR RT Master Mix with gDNA Remover, using RNA solution resulted from RNA extraction protocol, Nuclease-free water was added to RNA template. Thermal cycler condition was 65°C for 5 minutes, and after rapid cooling on ice, 2µl of 4×DN Master Mix (gDNA Remover) were added. The thermal cycler profile was 37°C for 10 minutes, then 2µl of 5×RT Master Mix were added to the mix. The thermal cycler condition for the 3rd step of PCR (reverse transcription reaction) was 37°C for 15 minutes, 98°C for 5 minutes. cDNA was used as template for real-time PCR analysis.

2.2.4. Quantitative RT-PCR for determining gene expression

A qRT-PCR on cDNA specimens was performed using SYBR Green Master Mix (Applied Biosystems). Elongation factor EF-1α gene was used as an internal control. The EF-

1α and their respective primers are presented in Table 1. All PCR reactions were performed in a reaction mixture containing 10.4µL of SYBR Green Master Mix, 4µL of 10pM primer set (ALF/EF-1α), 2µL of template DNA(10ng), and 3.6µL of nuclease-free water.

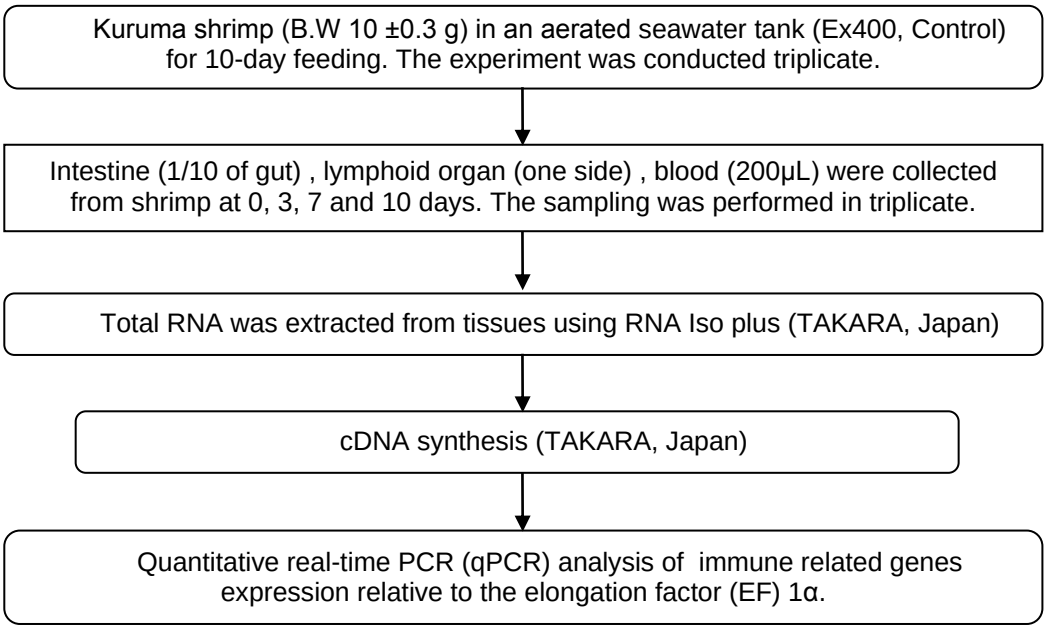

Diagram of experimental design

Table 1. The primers used to amplify EF-1 α and ALF of Kuruma shrimp

Gene	Sequence (5' to 3')	Accession number
[a]EF-1 α- Forward	TTCGCTGAACTGCTGACCAA	AB458256
[a]EF-1 α- Reverse	GCTTGCTGGGAACCATCTTG	
ALF- Forward	CCAACGCCCAACCTTCTACA	AB453738
ALF- Reverse	GGCTGCGGGTCATAGATCTG	

Note: [a] EF-1α specific primer were taken from a previous publication

Source: Maeda et al., 2014

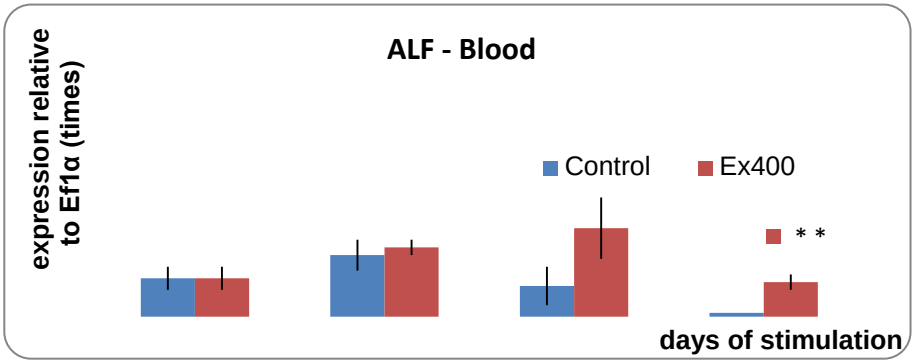

Fig.1. Quantitative real-time PCR analysis of ALF expression relative to (EF)-1α gene trancript in blood of control and Ex400 diet-fed Kuruma shrimp

*Note: Data are presented as mean ± SD. Differences were considered significant at P<0.05 and P<0.01 as indicated by asterisk * and **, respectively*

Amplification was carried out as follows: 60s at 95°C, 40 cycles of 15s at 95°C, and 40s at 60°C. Thermal cycling and fluorescence detection were conducted using RT-PCR system (Applied Biosystem) with detection run in duplicate. The threshold cycle (C_T) representing the PCR cycle at which an increase in reporter fluorescence above signal was first detected. The comparative C_T method $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) was used to analyze the expression level of the shrimp genes.

2.3. Statistical analysis

Analysis of variance was carried out using SPSS statistics version 18, to see the significance of expression of the gene at various time points. Independent t-test was performed to see significance in expression between Ex400 fed shrimp and control diet-fed shrimp.

3. RESULTS

3.1. Gene expression of ALF in blood

After 0, 3, 7 and 10 days of feeding, samples from 12 shrimps in each tank (Ex400 and control) were collected for checking expression by real time PCR. The levels of ALF lever in control group and experimental group (added Ex400 of the Ex supplementation) are shown in *Fig.*1. At 3 and 7 days post-feeding, although higher expression of ALF was observed in the blood of Ex400-fed shrimps, the expression levels was not different (P>0.05) from the expression in control diet-fed shrimp. ALF transcripts were significantly increased by Ex400 supplementation compared to control diet-fed shrimp (P<0.01) at 10 days post feeding. However, the level of expression of ALF in blood of both control diet-fed shrimp and Ex400 diet-fed shrimp at 10 day were lower than that 0 day post-feeding.

3.2. ALF expression in intestine

After 0, 3, 7, and 10 days of feeding experiment, intestine from 12 shrimps in each tank (Ex400 and control) were collected. The levels of ALF expression of control (without Ex400) and Ex400 of the Ex supplementation are given in Fig.2. Transcriptions of ALF were significantly increased by Ex400 in comparision to control diet fed-shrimp (with P<0.01) at 3 days post-feeding in the intestine. At 7 and 10 days post-feeding, although higher expression of ALF was observed in the intestine of Ex400-fed shrimps, the expression levels was not high from the control diet-fed shrimp.

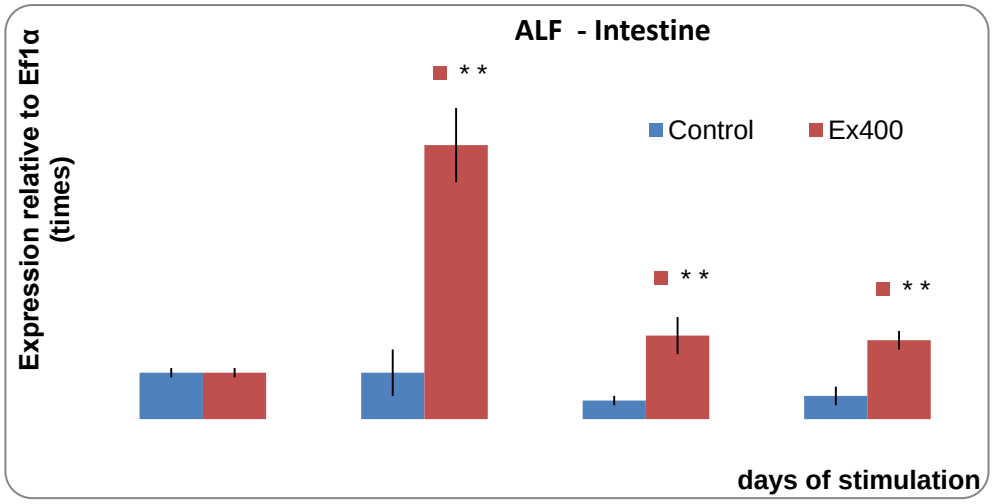

Fig. 2. Quantitative real-time PCR analysis of ALF expression relative to (EF)-1α gene trancript in the intestine of control and Ex400 diet-fed kuruma shrimp

*Note: Data are presented as mean ± SD. Differences were considered significant at P<0.05 and P<0.01 as indicated by asterisk * and **, respectively*

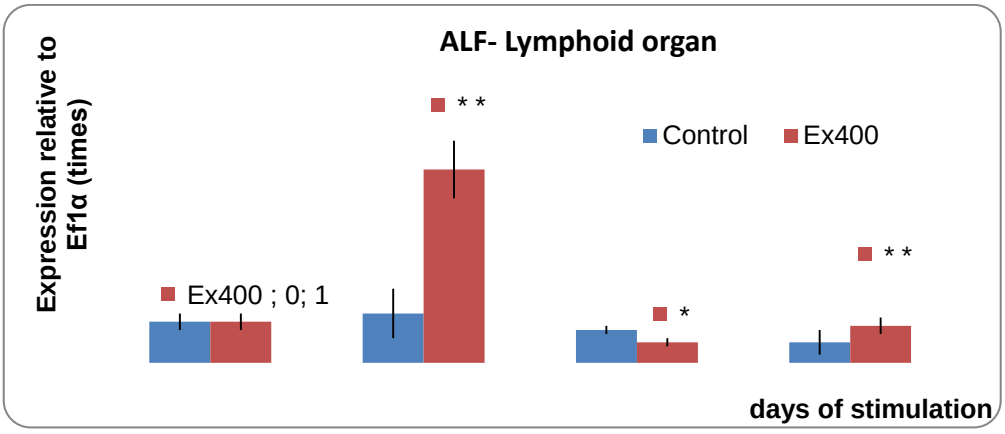

Fig. 3. Quantitative real-time PCR analysis of ALF gene expression relative to (EF)-1α gene trancript in the lymphoid organ of control and Ex400 diet-fed kuruma shrimp

*Note:Data are presented as mean ± SD. Differences were considered significant at P<0.05 and P<0.01 as indicated by asterisk * and **, respectively*

3.3. ALF expression gene in lymphoid organ

After 0, 3, 7 and 10 days of feeding, samples from 12 shrimps in each tank (Ex400 and control) were collected for checking expression by real time PCR. The levels of ALF lever in control group and experimental group (added Ex400 of the Ex supplementation) are shown in *Fig.*3. In the lymphoid organ, the transcript level of ALF in Ex400-fed shrimp was significantly higher (P<0.01) than control diet-fed shrimp at 3 days post feeding. ALF transcripts were significantly increased by Ex400 supplementation compared to control diet-fed shrimp (P<0.01) at 10 days post feeding, but the level of ALF expression was lower than that of 0 day.

4. DISCUSSION

The intestine was a favorable site for invasion of pathogens carried in the water, food, and sediment (Jayabalan *et al.*, 1982). It was previously demonstrated that an influx of hemocytes entered the intestine of *Penaeus monodon* following exposure to *Vibrio harveyi*. Besides, the hemocytes associated with the basal lamina of *S. ingentis* were reported to fight pathogens entering the body via the midgut (Liuxy *et al.*, 1996). Therefore, intestine which have immune functions in immune system effectively protects against pathogens. The lymphoid organ, first described in *Penaeus orientalis* (Oka, 1969), which consists of folded tubules with a central hemal lumen and a wall, layered with cells, was a site of bacterial uptake and phagocytosis by hemocytes (Van de Braak *et al.*, 2002). In most crustaceans, such as crabs and lobsters, that do not possess the lymphoid organ, phagocytes are involved in the uptake of foreign materials (Johnson, 1987). However, in those that do possess a lymphoid organ, including shrimps, it is the main site of bacteriostasis (Burgents *et al.*, 2005). These results are consistent with our study because we found that ALF was significantly increased after 3 days post feeding in intestine and lymphoid organ. Besides, at 3 and 7 days post-feeding, although higher expression of ALF was observed in the blood of Ex400-fed shrimps, the expression levels were not different (P>0.05) from the control diet-fed shrimp, and the levels of expression of ALF of both control diet-fed shrimp and Ex400 diet-fed shrimp at 10 day were lower than that 0 day post-feeding in blood. This suggestion may be supported by the hypothesis of Beale who attributed the increase in ALF gene expression in tissue to the higher concentration of the haemocytes following bacterial infection (Beale *et al.*, 2008)

Regarding the effect of immuno-stimulant on the gene expression of the immune-related genes. ALF gene expression showed significant increase (eight fold) on the 3rd day, which followed by sharp decrease nearly towards the control on the day seven (El-Asely *et al*., 2011). The effect of the immuno-stimulants on the ALF gene expression was recorded by Mekata who observed the highest expression of ALF at 48, 8 and 12 h after lipopolysaccharide (LPS) injection at 1, 10 and 100 μg, respectively (Mekata *et al*., 2010). Other works manipulated the expression of the ALF following bacterial challenge where they showed an increase in its expression short time after challenge (Beale *et al*., 2008). The sharp increase of ALF gene expression recorded on the day three following administration of MACH may be associated with the fast and significant increase in the THC at the highest dose 0.2% MACH fed shrimp (El-Asely *et al*., 2011).

In addition, the Ex400 diet test containing Euglena and microalgae produced Polysaccharide and beta-glucan. Polysaccharide is produced by microalgae and applied as anti-virus agent, antioxidant, anti inflammation and as part of immunomodulatory system. Beta-glucan is able to activate phagocytes effectively in invertebrates. According to a previous study, shrimp fed with peptidoglycan-supplemented feed showed better growth and feed conversion rates than those fed a normal diet, and demonstrated that black tiger shrimp grew faster with glucan immersion which could be attributed to the higher activity of glucan delivered by immersion compared to oral administration (Boonyaratpalin *et al*., 1995).

In this context, we observed the up-regulated ALF transcription in intestine, lymphoid organ of Ex400-fed shrimps at 3 days post-feeding. This result coupled with our findings, therefore, indicated that ALF is elicited by immune-stimulating substances and acts as an integral component of the shrimp antibacterial defense mechanism.

Based on the results obtained, it will be of great interest to determine the gene expression such as Crustins, Penaeidins, Toll receptor in Kuruma shrimp in response to an in *vivo* stimulation, and its resistance against viruses or bacteria.

5. CONCLUSIONS

ALF transcripts were significantly increased by Ex400-fed shrimps compared to control diet-fed shrimps ($P< 0.01$) at 3 post-feeding in lymphoid organ and intestine. The ALF gene expression could activate ALF in the immune system of Kuruma shrimp and suggested that Ex400 has a potential use as immuno-stimulant for shrimps.

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FACTORS AFFECTING RESIDENTIAL LAND PRICE IN DIEN BIEN PHU CITY, DIEN BIEN PROVINCE

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Received date: 18.01.2017 Accepted date: 25.09.2017

ABSTRACT

The purpose of this study was to apply multivariate linear regression model to determine the factors that affect residential land price in Dien Bien Phu city, Dien Bien province. The obtained results showed that there were four groups of factors affecting the residential land price in Dien Bien Phu city, including: social factors, economic factors, regional factors and individual factors. Collected data were processed using the statistical software SPSS; the consistency coefficient was measured with Cronbach's Alpha for scaling test and Exploring Factor Analysis (EFA) was used. The analysis of Multivariate Linear Regression Model indicated that 61.1% of the variation of residential land price could be explained by the variation of the 4 groups of independent variables, while the rest (38.9%) was explained by other factors outside the model. The model that showed factors affecting the residential land price of Dien Bien Phu city as follow: $Y = 0.644 + 0.185X_1 + 0.261X_2 + 0.493X_3 + 0.327X_4$. The regional factor had the highest effect on residential land price with 23.9% contribution, followd by individual factor with contribution of 15.78%, economic factor with contribution of 12.6%, and social factor with contribution of 8.93%.

Keywords: Affect, Dien Bien Phu city, factor, residential land prices.

Nghiên cứu các yếu tố ảnh hưởng đến giá đất ở trên địa bàn thành phố Điện Biên Phủ, tỉnh Điện Biên

TÓM TẮT

Mục tiêu của nghiên cứu này là ứng dụng mô hình hồi quy tuyến tính đa biến để phân tích các yếu tố ảnh hưởng đến giá đất ở trên thị trường của thành phố Điện Biên Phủ, tỉnh Điện Biên. Kết quả nghiên cứu cho thấy, có 4 nhóm yếu tố tác động đến giá đất ở tại thành phố Điện Biên Phủ gồm: nhóm yếu tố khu vực, nhóm yếu tố cá biệt, nhóm yếu tố xã hội và nhóm yếu tố kinh tế. Số liệu được xử lý bằng phần mềm SPSS, kiểm định thang đo bằng hệ số Cronbach's Alpha và mô hình phân tích nhân tố khám phá EFA. Kết quả phân tích hồi quy tuyến tính đa biến cho thấy 61,1% sự biến động của giá đất bị ảnh hưởng bởi các nhóm yếu tố đưa vào mô hình nghiên cứu, còn lại 38,9% sự biến động của giá đất là do các yếu tố khác. Phương trình hồi quy tuyến tính về các yếu tố ảnh hưởng đến giá đất có dạng $Y = 0,644 + 0,185X_1 + 0,261X_2 + 0,493X_3 + 0,327X_4$, trong đó, yếu tố khu vực được xác định là yếu tố ảnh hưởng lớn nhất đến sự biến động của giá đất với sự đóng góp là 23,9%, tiếp đến là yếu tố cá biệt (15,78%), yếu tố kinh tế (12,6%) và cuối cùng là yếu tố xã hội (8.93%).

Từ khóa: Ảnh hưởng, giá đất ở, thành phố Điện Biên phủ, yếu tố.

1. INTRODUCTION

The rapid expansion of cities in the developing countries due to urbanization, population growth and economic development creates numerous problems. Besides the land demand for construction of residential buildings, commercial centers make the land

resource becoming scarce and lead to an increase in residential land price. Consequently, low-income people are very difficult to access this scarce resource.

The land price is determined by the economic principle of highest and best use of land which produces the highest net return in any term, over a period of time. The lack of reliable nationwide databases on land transactions also makes it difficult to estimate land price exactly. Study of factors affecting land price is essential for calculating or estimating land price.

Studying factors affecting residential land price has been done by many researchers. Several studies showed that distance from the Central Business District is the major determinant of land price while the effects of non-location factor like plot size, time of land purchase, age of neighborhood, income, zoning policy, etc. are neglected (Alonso, 1964; Ball, 1973; Asabere, 1982). Another studies showed that age, location, size, neighborhood characteristics, economic activity, population, transport, etc are factors affecting land price (Asabere and Huffman, 1996; Kauko, 2003;, Joslin, 2005). Moreover, land value does not only depend on the physical characteristics of a building but also the environment that surrounds the building (Lancaster, 1966; Topcu and Kubat, 2009).

Dien Bien Phu city is the political, administrative, economic and cultural center of Dien Bien province, with a total area of 6,427.10 hectareas (Department of Natural Resources and Environment of Dien Bien Phu city, 2015). National Highway 12 and Highway 279 running through Son La and Lai Chau are favorable conditions for developing economy and society and expanding exchanges with the neighboring districts and provinces. According to Lo Thi Hong (2016), the residential land prices regulated by the State (benchmark) has been increased significantly in Dien Bien Phu city, especially in the period of 2013 - 2015 (average increase from 8 to 10% per year). There has been a large difference between benchmark price and market price (from 1.05 to 5 times in the urban area; from 1.1 to 2.18 times in the rural area). The purpose of this study was to apply multivariate linear regression model to determine the factors that affect residential land prices in Dien Bien Phu city, Dien Bien province.

2. THE HYPOTHESIS AND METHODOLOGIES

2.1. Hypothesis

Based on the previous studies and characteristics of the study site, a research scheme was developed (Figure 1). All social, economic, regional and individual factors were hypothesized to have a positive influence on residential land price of Dien Bien Phu city.

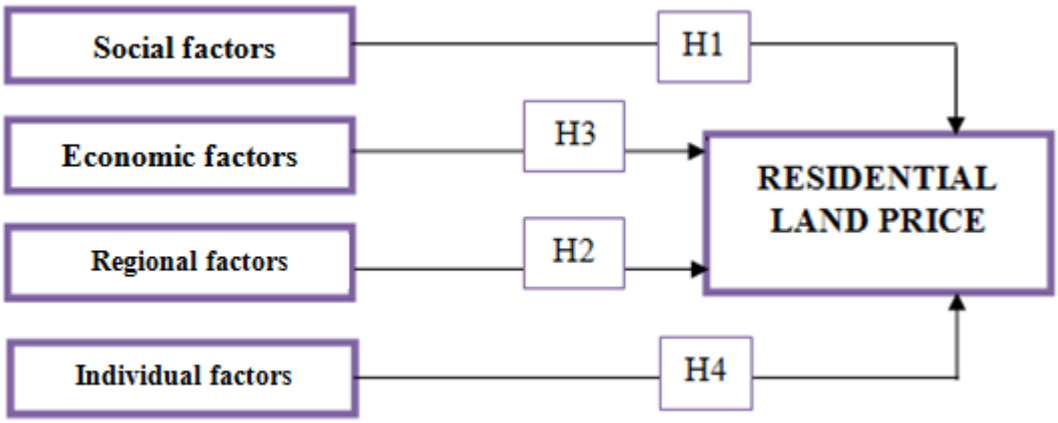

Figure 1. The research scheme

2.2. Methodologies

2.2.1. Data collection

Secondary data on the current of land use and land price of Dien Bien Phu city were collected from Department of Natural Resources and Environment and People's Committee of Dien Bien Phu city.

Primary data were gathered in the form of responses from the respondents. The five-level Likert scale of 1 to 5 was used to design the questionnaire form on measuring the influence of the factors affecting the residential land price, where 5 = strongly influence, 2 = influence, 3 = neutral, 4 = less influential and 5= not influential) (Likert, 1932). The respondents were required to choose only one option for every question. The average was calculated for each statement. The result was then concluded with an overall average. The sample size was determined based on requirements of Exploratory Factor Analysis and Regression Analysis (Hoang Trong Chu and Nguyen Mong Ngoc, 2005). The minimum number of samples of the study was 150 participants. However, based on the number of land use right transfer in Dien Bien Phu city, the study investigated 185 participants (30 officials who work in land management field and real estate brokers and 155 people involved in buying or selling land).

In order to test the reliability of the measuring data, the Cronbach's Alpha and Exploratory Factor Analysis were applied. The factors affecting the residential land price were analyzed by multivariate regression model. Statistical Package for Social Science (SPSS) version 16.0 was used for analysis and presentation of the result. The descriptive statistics such as frequency tables was applied to analyze data.

2.2.2. Data analysis

- Cronbach's alpha reliability test: Alpha was developed by Lee Cronbach in 1951 to provide a measure of the internal consistency of a test or scale (Cronbach, 1951). There are different reports about the acceptable values of alpha, ranging from 0.70 to 0.95 (Bland, Altman, 1997; Nunnally and Bernstein,1994). A low value of alpha could be due to a low number of questions, poor interrelatedness between items or heterogeneous constructs. If alpha is too high it may suggest that some items are redundant as they are testing the same question but in a different guise. A maximum alpha value of 0.90 has been recommended (Streiner, 2003). Outside Cronbach's alpha, Corrected Item - Total Correlation is also used to test the data reliability. The data will be accepted when Corrected Item - Total Correlation > 0.3 (Nunnally & Bernstein, 1994; Hair *et al.,* 1998). A correlation value less than 0.2 or 0.3 indicates that the corresponding item does not correlate very well with the scale overall and, thus, it may be dropped (Field, 2005).

- Exploratory Factor Analysis (EFA): EFA was used to identify the underlying relationships between measured variables (Norris *et al.,* 2010). Parameters applied were index of Kaiser-Mayer-Olkin (KMO), Bartlett's test, coefficient of eigenvalues, total variance explained and factor loading. The variables were accepted when KMO (Kaiser - Meyer - Olkin) ranges from 0.5 to 1.0 and Factor Loading is less than 0.35 or the distance between two of Factor Loading of one variable in 2 different factors is greater than 0.3 (Igbaria *et al.,* 1995); Total Variance Explained is also larger than 50%; Bartlett's coefficient at sig <0.05; Eigenvalue coefficient value ≥ 1 (Kaiser, 1960).

- Multivariate regression analysis was used to estimate the factors that affecting the residential land price. The following function was developed: $\beta o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + ... + \beta_n X_n + Ei$

Where:

- Y_i : dependent variable represents the land price.

- X_1; X_2; X_3; X_4; X_n: independent variables representing factors affecting the land price.

- βo: regression intercept

- β_1, β_2, β_3, β_4: regression coefficients

-E_i: standard error.

- n: Number of variables.

Statistical Package for Social Science (SPSS) version 16.0 was used for analysis and presentation of the result. The descriptive statistics such as frequency tables was applied to analyze data.

3. RESULTS AND DISCUSSION

3.1. Identifying the factors affecting residential land price in Dien Bien Phu city

There were many factors that affect residential land prices. The key among these included psychological and social factors (security and crime rate, psychological, spiritual, tastes, urbanization, population density, speculate), economic factors (economic development speed, income and expenditure, price variation, interest rate of bank, reserve, investment), regional factors (location, Infrastructure, environmental quality, plan), individual factors (area, facade width, land plot depth, land shape, slope, urban planning limitation), legal factors (certificate of land use rights, land allocation decision), international factors (world economy, world politics), market related factors (supply, demand, supply and demand relation), state and law factors (financial and credit policy, tax policy, investment policy, land policy) (Ho Thi Lam Tra and Nguyen Van Quan, 2006; Ho Thi Lam Tra *et al.*, 2017), and land accessibility to amenities and services (schools, health, shopping, recreation, and other services). However, only 4 groups (society, economy, region and individuality) with 20 factors were selected for the study. These were factors that significantly affect the current downturn in the residential land price of Dien Bien Phu city (Table 1). These factors have a close relationship with variation of the real estate market in general and the land market in particular. Among the regional factors, the location was the major determinant factor affecting the value of a piece of land. As a rule, the closer a piece of land is to a population center, the greater is its value. Among the social factors, social security, population density and urbanization were determinant factors. The higher population density creates more demand, which increases the competition for a piece of land and the price that buyers are willing to pay for it. For land in sparsely populated areas, even better land, fewer people are willing to pay for it. In addition, more desirable locations within densely populated areas command higher prices. Among the individual factors, the facade width and shape had the greatest impact on the value of land plots. Among the economic factors, the residential land price was affected by economic development speed, income and expenditure. In Dien Bien Phu city, the highest residential land price was recorded for Vo Nguyen Giap street (road near the Center market and Victory Monument). This street is located near center of the city and has high population density.

3.2. Cronbach's alpha reliability test and Exploratory Factor Analysis

3.2.1. Cronbach's alpha reliability test

The result of data analysis by SPSS software determided the Cronbach's Alpha, total correlation and the Cronbach's Alpha if Item Deleted (Table 2). All of the Cronbach's Alpha coefficients of observed items were greater than 7. This implied that all data ensure the reliability. Results of analyzing the Corrected Item - Total Correlation in the column 2 identifed 2 variables including slop (IN6) and urban planning limitations (IN7) had a value less than 0.3. This implied that these 2 variables were not eligible for further analysis.

3.2.2. Exploratory Factor Analysis

The results of Exploratory Factor Analysis for independent variable are as follow:

- Kaiser-Meyer-Olkin (KMO) and Bartlett's Test: KMO and Bartlett's Test of Sphericity is a measure of sampling adequacy that is recommended to check the case to variable ratio for the analysis being conducted. In most academic and business studies, KMO and Bartlett's test play an important role for accepting the sample adequacy.

Table 1. Factors affecting the price of land in Dien Bien Phu city

N^0	Factor	N^0	Factor
I	Group of social factors	10	Location
1	Security	11	Infrastructure
2	Psychological, spiritual, tastes	12	environmental quality
3	Urbanization	13	Plan
4	Population density	IV	Group of individual factors
5	Educational level	14	Shape
II	Group of economic factors	15	Area
6	Economic development speed	16	Land navigation
7	Income and expenditure	17	Facade width
8	Price variation	18	Land plot depth
9	Interest rate of Bank	19	Slope
III	Group of regional factors	20	Urban planning limitation

Table 2. Results of analysis Cronbach's Alpha

N^0	Influencing factos	Symbol	Corrected Item - Total Correlation	Cronbach's Alpha if Item Deleted
I	Group of social factors (Cronbach's Alpha = 0,849)			
1	Security	SO1	0.67	0.81
2	Psychological, spiritual, tastes	SO2	0.67	0.82
3	Urbanization	SO3	0.64	0.82
4	Population density	SO4	0.65	0.85
5	Educational level	SO5	0.66	0.82
II	Group of economic factors (Cronbach's Alpha = 0,793)			
6	Economic development speed	EC1	0.60	0.75
7	Income and expenditure	EC2	0.63	0.73
8	Price variation	EC3	0.60	0.74
9	Interest rate of Bank	EC4	0.60	0.75
III	Group of regional factors (Cronbach's Alpha = 0,820)			
10	Location	RE1	0.68	0.76
11	Infrastructure	RE2	0.62	0.78
12	Environmental quality	RE3	0.65	0.77
13	Plan	RE4	0.62	0.79
IV	Group of individual factors (Cronbach's Alpha = 0,706)			
14	Shape	IN1	0.63	0.62
15	Area	IN2	0.60	0.62
16	Land navigation	IN3	0.62	0.65
17	Facade width	IN4	0.60	0.63
18	Land plot depth	IN5	0.58	0.63
19	Slope	IN6	0.06	0.76
20	Urban planning limitation	IN7	-0.02	0.77

The KMO statistic is a measure of sampling adequacy, both overall and for each variable. The KMO statistic varies between 0 and 1. The value of KMO is more than 0.7 that is the common threshold for confirmatory analysis (Hair *et al.,* 2010). Kaiser (1974) recommends that the value of KMO is greater than 0.5 as acceptable. Furthermore, values between 0.5 and 0.7 are considered mediocre, values between 0.7 and 0.8 are considered good, values between 0.8 and 0.9 are deemed great and values above 0.9 are superb (Hutcheson and Sofroniou, 1999). For these data the value was 0.8, which falls into the range of being deemed great. So, we should be confident that factor analysis was appropriate for these data.

The Bartlett's test of Sphericity relates to the significance of the study and thereby shows the validity and suitability of the responses collected to the problem being addressed through the study. For Factor Analysis to be recommended suitable, the Bartlett's test of Sphericity must have a significance value less than 0.05. Results of the test done by SPSS software showed that the Bartlett's test of Sphericity have significant value at the 0.000. So, for these data, Bartlett's test was highly significant, and therefore factor analysis was appropriate.

- Factor extraction: The purpose of extracting the factor is determining the linear combination of variables that account for the greatest amount of common variance. Data from table 3 lists the eigenvalues associated with each linear factor before extraction, after extraction and after rotation. According to Kaiser (1960), we can retain only factors with Eigenvalues greater than 1 and Total Variance Explained is also larger than 50%. The result in table 3 showed that the first factor accounted for the greatest amount of common variance (23.037%), representing an eigenvalue of 4.147. Each subsequent factor explained a portion of the remaining variance until a point is reached where it can be said that the factors no longer contribute to the model. At this point, those factors with an Eigenvalues above 1 present the number of factor needed to describe the underlying dimensions of the data. In this study, this was factor 4, with an explained variance of 9.654 and Eigenvalues of 1.736. All of the factors with Eigenvalues were smaller than 1 that did not contribute and adequate amount to the model to be included. This implied they were not correlated with each other. Thus, only 4 factors (1-4) contributed to the model.

- Factor Loading: Factor loading is the correlation between a variable and a factor where only a single factor is involved or multiple factors are orthogonal. In general, the data is confident if Factor Loading is greater than 0.3. However, higher Factor Loadings indicate that variable is closely associated with the factor. It also contributes to construct validity (Hair *et al.,* 2010). The results obtained in the matrix of correlation in Table 4 showed that all the variables had high degree of positive relationship with one another. They have in the range from 0,739 to 0,822. Thus, all Standardized Factor Loadings in our model were significant; this was a confirmation of the validity of the theoretical framework. The score on the relationship between accessibility and location showed the highest positive associated with a figure of .822. This means that location was the most determinant factor affecting the residential land price in Dien Bien Phu city.

The results of Exploratory Factor Analysis for dependent factors (SO, EC, RE, IN) were also identified KMO coefficient, the Total Variance Explained of 0.738 and 60.87 respectively; Bartlett test values significantly (sig <0.05), the coefficient of Eigenvalues> 1; Factor Loadings were 0.828; 0.780; 0.764; 0.747. Thus, the dependent variables were also eligible this study.

Table 3. Total varience Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of variance	Cumulative %	Total	% of variance	Cumulative %	Total	% of variance	Cumulative %
1	4.147	23.037	23.037	4.147	23.037	23.037	3.308	18.375	18.375
2	3.274	18.187	41.224	3.274	18.187	41.224	3.171	17.616	35.991
3	2.502	13.901	55.125	2.502	13.901	55.125	2.654	14.746	50.737
4	1.736	9.645	64.770	1.736	9.645	64.770	2.526	14.032	64.770
5	.770	4.279	69.049						
6	.607	3.371	72.419						
7	.551	3.064	75.483						
8	.544	3.021	78.504						
9	.533	2.959	81.462						
10	.516	2.865	84.328						
11	.495	2.749	87.077						
12	.448	2.487	89.563						
13	.385	2.139	91.702						
14	.366	2.032	93.734						
15	.333	1.850	95.584						
16	.294	1.632	97.216						
17	.277	1.540	98.756						
18	.224	1.244	100.000						

Table 4. Rotated Component Matrix

Variable	Component			
	1	2	3	4
IN1	.818			
IN2	.805			
IN3	.797			
IN4	.790			
IN5	.761			
SO1		.809		
SO2		.795		
SO3		.785		
SO4		.772		
SO5		.760		
RE1			.822	
RE2			.813	
RE3			.753	
RE4			.739	
EO1				.789
EO2				.786
EO3				.785
EO4				.775

3.3. Applied multivariate regression analysis to estimate the influence level of factors that affecting the residential land price in Dien Bien Phu city

In this section, stepwise multiple regression analysis was computed at significant level of (p=0.05) in order to examine which factors could be affected residential land price in Dien Bien Phu city. The result of running regression model determined adjusted R^2 is 62.1% and R^2 = 61,1%, it means that 61.1% of the variation of residential land price could be explained by the variation of the 4 groups of independent variable, while the rest (38.9%) was explained by other factors outside the model. Table 5 showed the value of Durbin-Watson of 1,838. The Durbin - Watson statistic (d) is a test statistic used to detect the presence of autocorrelation in the residuals (prediction errors) from a regression analysis. The value of d always lies between 0 and 4. A value near 2 indicates non-autocorrelation; a value toward 0 indicates positive autocorrelation; a value toward 4 indicates negative autocorrelation. Therefore, we can assume that there was no first order linear auto-correlation in our multiple linear regression data.

Regression Coefficients is presented in table 5 indicated that a relatively high percentage of the variation in the residential land price could be explained by the variables. As it was indicated in the table 5, Sig. = 0,000 that was less than the significant level (0,01) for all variables. This implies that all variables have significant impact on residential land price and there was significant association between independent and dependent variables. Through the Standardized Beta Coefficient, we determined the importance of each variable in the regression model or the impact level of each independent variable on the dependent variable. The Standardized Beta Coefficient of regional variable had the highest value (0.493), this implied that regional factor had the highest effect on the residential land price in Dien Bien Phu city. The study results also showed that 1 unit changes of social factor would led to the residential land price changes 0.185 unit, 1 unit changes of economic factor would led to residential land price changes 0.261 unit, 1 unit changes of regional factor l led would to residential land price changes 0.493 unit, 1 unit changes of individual factor would led to residential land price changes 0.327 unit.

The panel model of residential land price could be expresses as following:

$$Y = 0.644 + 0.185X_1 + 0.261X_2 + 0.493X_3 + 0.327X_4$$

From the standardized beta coefficient, we could change to percent ratio, of which social variable contributed 8.93%, economic variable contributed 12.60%, regional variable contributed 23.79.93% and individual variable contributed 15.78% (Table 6).

Analysis of variance (ANOVA) was used to test the reliability of the regression analysis. The result of table 7 showed that F value = 61.382. The F-test is highly significant, thus we could assume that there was a linear relationship between the variables in our model. Beside, the value of Sig (P-value) of the ANOVA tables used to assess the suitability (N) of the model. The value of Sig is small (<5%), the model was suitable. In summary, the result of regression analysis ensured reliability.

Table 5. Regression Coefficients

Model	Unstandardized coefficient	Standardized coefficient	Sig.	Collinearity Statistics	
	β	Beta (β)		Tolerance	VIF
(Constant)	0.644		0.002		
X_1 - Society	0.105	0.185	0.000	0.946	1.057
X_2 - Economy	0.178	0.261	0.000	0.973	1.028
X_3 - Region	0.312	0.493	0.000	0.852	1.173
X_4 - Individual	0.187	0.327	0.000	0.906	1.104

Table 6. The effect of the factors on residential land prices in Dien Bien Phu city

Factors	Standard.Beta	Ratio (%)	Order
X_1 - Society	0.185	8.93	4
X_2 - Economy	0.261	12.60	3
X_3 - Region	0.493	23.79	1
X_4 - Individual	0.327	15.78	2
Total	1.266	61.10	

Table 7. Analysis of variance ANOVA

Source	Sum of Squares (SS)	Degree of Freedom (D.f)	Mean of Squares	F ratio	Sig.
Between Group	19.861	4	4.965	61.296	.000[a]
Within Group	12.134	150	0.081		
Total	31.994	154			

4. CONCLUSION

It is evident from the study that, there were 4 groups of factor that affecting the residential land price in the market of Dien Bien Phu city. They were including of social factors, economic factors, regional factors and individual. There was significant and positive relationship between these factors and the residential land price. The results of analysis of Multivariate Linear Regression Model indicate that 61.1% of the variation of residential land price could be explained by the variation of the 4 groups of independent variable, while the rest (38.9%) was explained by other factors outside the model. The model that showed factors affecting the residential land price in the market of Dien Bien Phu city as follow: $Y = 0.644 + 0.185X_1 + 0.261X_2 + 0.493X_3 + 0.327X_4$. The regional factor had the highest effect on the residential land price, it contributed 23.9%; the second was individual factor with contribution of 15.78%; the third was economic factor with contribution of 12.6%; the final was social factor with contribution of 8.93%.

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Vietnam J. Agri. Sci. 2017, Vol. 15, No. 9: 1196- 1204 Tạp chí Khoa học Nông nghiệp Việt Nam 2017, 15(9): 1196 - 1204
www.vnua.edu.vn

BUILDING A DATABASE FOR FINANCIAL MANAGEMENT OF LAND IN NINHHIEP COMMUNE, GIALAM DISTRICT, HANOI CITY

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Received date: 10.08.2017 Accepted date: 02.10.2017

ABSTRACT

Gialam district is one of the gateways into Hanoi capital, where urbanization is actively progressing. As a result of urbanization, in recent years, land market in Gialam has been booming. Therefore, in order to avoid possible financial losses on land use, it is necessary to improve the cadastral management system. A cadastral database has been developed for Ninhhiep commune, Gialam district based on GIS technology, which includes spatial data (landuse maps) and their attributes (detailed information of land owner, land category, land price and legal status of 3494 land parcels). This properly and carefully developed cadastral database allows users to g*et al*l necessary information (referring map and its attributes) for every land parcel in the area. Therefore, while using this cadastral database, users can get recorded land use status, land use ID and its area, and land use owner's financial responsibility. This cadastral database also allows users to get necessary information for preparing financial reports on land use and to develop a land price map. It can also support land use owners to clarify their financial responsibility. In order to enable timely update land use information, WebGIS technology was integrated in the database to allow users to get necessary land use information through internet (with limited access to secure this cadastral database) using smart phones. The result of the present study is practically significant in the era of the development of information technology for management activities in general and for cadastral management in particular.

Keywords: Database, land use, land price, financial management of land, cadastral management.

Xây dựng cơ sở dữ liệu phục vụ công tác quản lý tài chính về đất đai tại xã Ninh Hiệp, huyện Gia Lâm, thành phố Hà Nội

TÓM TẮT

Huyện Gia Lâm là cửa ngõ vào thủ đô Hà Nội đang trong quá trình đô thị hóa. Trong những năm gần đây, thị trường đất đai ở Gia Lâm trở nên sôi động nên công tác quản lý đất đai cần được đẩy mạnh nhằm tránh tổn thất về tài chính đất đai. Với sự trợ giúp của công nghệ GIS, các nhà nghiên cứu đã xây dựng cơ sở dữ liệu, bao gồm cơ sở dữ liệu dữ liệu không gian (bản đồ thửa đất) và dữ liệu thuộc tính bao gồm các thông tin về chủ sử dụng đất, loại đất, giá đất và tình trạng pháp lý cho 3494 thửa đất. Dữ liệu không gian và dữ liệu thuộc tính của thửa đất đã được xây dựng một cách đầy đủ,chi tiết, đồng nhất, trung thực và có một liên kết Web tốt. Sau khi hoàn thành cơ sở dữ liệu, cơ sở dữ liệu có thể hiển thị các thông tin thuộc tính như: tình trạng sử dụng đất, mã thửa đất và diện tích, cũng như tình trạng của các nghĩa vụ tài chính; cho phép Tìm kiếm, tổng hợp thông tin tài chính về đất đai; Hỗ trợ thủ tục kê khai các nghĩa vụ tài chính về đất đai; Xây dựng bản đồ giá đất; Để cập nhật cơ sở dữ liệu của bất kỳ thay đổi một cách kịp thời. Nghiên cứu cũng sử dụng công nghệ WebGIS để đưa toàn bộ thông tin về cơ sở dữ liệu trên internet (có phân quyền bảo mật) để cung cấp thông tin về cơ sở dữ liệu và sử dụng điện thoại thông minh là một công cụ phổ biến để có thể truy cập cơ sở dữ liệu. Đây là một nghiên cứu có ý nghĩa thiết thực trong kỷ nguyên phát triển công nghệ thông tin cho tất cả các ngành nói chung và quản lý đất đai nói riêng.

Từ khóa: Cơ sở dữ liệu, giá đất, quản lý tài chính về đất đai, quản lý đất đai, sử dụng đất.

1. INTRODUCTION

Information technology is rapidly growing and it is predicted to continue the growing trend in the future. Information technology helps to solve a wide range of complicated socio-economic issues, thus, it is considered as an inevitable instrument for management. In order to meet the management requirements and exploit this advanced technology in land management, it requires strong changes in organizing as well as in improving quality of cadastral information.

At present, there are lots of gaps and obstacles in financial management of land, causing losses of large revenues for the state. The current manually paper-based data organisation, achieve and management system hardly meet the requirement of fast solving question-answer demands, causing difficulties in analysis and synthesis of information related to financial management of land. Therefore, cadastral management sector needs land related information and accurate data in a scientific arrangement for different purposes.

Ninhhiep is a commune of Gialam district, Hanoi City. Similar to other communes in the district, baseline survey data, paper-based maps, log-books, and record books are not unified and cumbersomely archived, leading to difficulty in information retrieval and obstacles in land management in general and financial management of land in particular. Therefore, building a database that serves the financial management of land in the commune and its expansion to a district-wide database for financial management of land in the future is an urgent need.

2. MATERIALS AND METHODS

2.1. Study area

Ninhhiep commune was selected for the present study. It is located in the northeast of Gialam district, about 18 km from the center of Hanoi. The commune has a very convenient geographic location, convenient transportation, and an eventful commodities exchange point in a stable political, cultural, and social status with good economic development. Land use and management, especially financial management of land should be strict monitored in order to generate income source for the state budget.

2.2. Methods

In the present study, cadastral maps of Ninhhiep commune were collected and used as a source of spatial data of the database. Cadastral documents including logbooks, cadastral books, logbooks of landuse right certificates (LURCs), monitoring books of land use changes, current status of land use in 2016, the decision announcing land price in 2014 by Hanoi People's Committee, and other cadaster-related information on the study area were collected from government agencies at different levels. These data were then used as a source of attribute data of the database.

Cadastral maps were then edited in MicroStation software and exported to Shapefiles in ArcGIS. 18 fields of the attribute database were created and information of all land parcels were added.

A number of applications based on the established database were then deployed using functions of ArcGIS such as mapping, calculating, analyzing and searching data.

Finally, ArcGIS Online and ArcGIS Server software were used to publish the database online. The new Web-page Application Builder was used to create a website for database sharing.

3. RESULTS

3.1. Spatial Database

In order to undertake spatial data, the collected cadastral maps were edited using MicroStation software to create a general cadastral map for Ninhhiep commune. The map was then exported to ArcGIS Desktop for editing to create the spatial database of the study area.

Research object has been classified into specific layers and transferred in Geodatabase on ArcCatalog for storing.

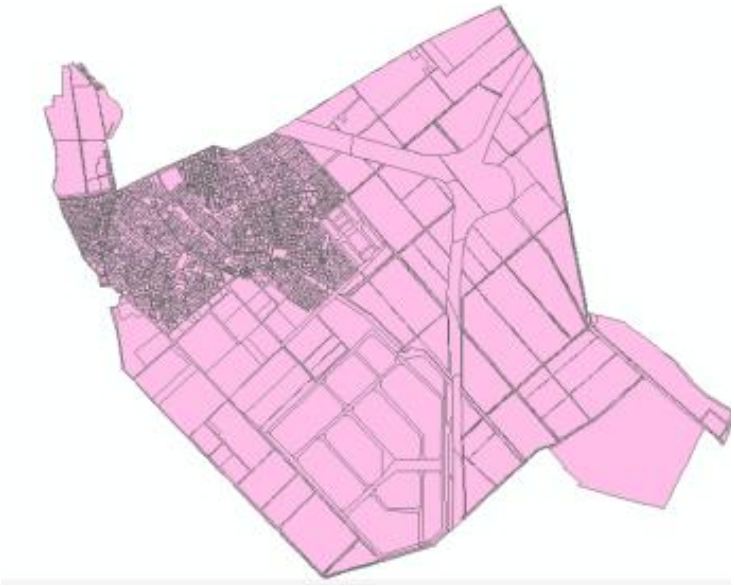

Figure 1. Database for land parcels in shape file *.shp

Table 1. Attribute data of land parcel layer

Field name	Field type	Remark
CHUSD	Text (50)	Name of the land use owner
NAMSINH	Float	Date of birth of the land use owner
TENDUONG	Text (50)	Street name
DIACHI	Text (40)	Address of the land parcel
VITRI	Float	Location of the land parcel
TOBANDO	Short Integer	Map number
THUASO	Text (3)	Land parcel number
DIENTICH	Float	Land parcel area
TINHTRANG	Text (40)	land use rights status
SO_GCN	Text (20)	Land use right Certificate number
NAMCAP	Text (10)	Issue date of Land use right Certificate
GIADATQD	Short Integer	Land price according to State Regulation [3]
NGUONGOC	Text (50)	History of land parcel
MDSD	Text (30)	ID of land parcel
LOAIDAT	Text (30)	Land use type
NVTC	Text (40)	Financial obligation status
NVTC_NO	Text (10)	Type of financial obligation in debt
GHI_CHU	Text (70)	Other remarks if neccesary

The data of the land parcels after converting are line data. Therefore, these data on the land parcels were converted to polygons using the Feature to Polygon tool in ArcToolbox. The resultant polygon data were then used to create the spatial database of Ninhhiep Commune (Figure 1).

3.2. Attribute database

The data collected included cadastral maps, books of accounts, logbooks for LURCs, cadastral books, land use monitoring books, registration and declaration books for landuse (LU) owners classified per street of the town, decisions on issuing LURCs, etc. These data were then

aggregated according to the data fields with each row storing attribute information of a parcel. In total, 18 data fields were created and attribute data of 3494 parcels of land in Ninhhiep commune were built (Table 1).

3.3. Application of the database for financial management of land in Ninhhiep commune

3.3.1. Look-up information on financial status of a land parcel

User of the database can look up (retrieve) information on a land parcel, or in other words, look up information by an independent value. The information can be looked up, such as map No., land parcel No., land use owner, and land use purpose, etc.

With the functions of searching and inputting information of land parcel, the retrieval result is the image of the land parcel on the map and its attribute data.

3.3.2. Information retrieval and aggregation on land finance

Using the attribute selection function, database users are able to search and aggregate all available financial data of all land parcels in the commune quickly and accurately in a few simple steps. This function significantly enhances the effectiveness of financial management of land in the area compared to the previous manual management (Figure 2).

3.3.3. Support in identifying financial land receipts

From the present land-price database, the amount of money to be paid by the land users to the state in the performance of financial obligations are calculated using query function and calculating function in combination with formulas for calculating financial obligations [4, 5], for instance:

- Calculation of land use fee must be paid upon receipt of a certificate of land use right:

*Land use fee (VND) = land area (m^2) * land price (VND/ m^2)*

- Calculation of personal income tax upon receipt of a certificate of land use right or land use right transfer (in the case income from land is unidentifiable)

Personal income tax (VND) = 2% land price (VND/ m^2) * land area (m^2)*

- Calculation of the registration fee upon receipt of a certificate of land use right or transfer of land use right

Registration fee = 0.5% land price (VND/ m2) * land area (m^2)*

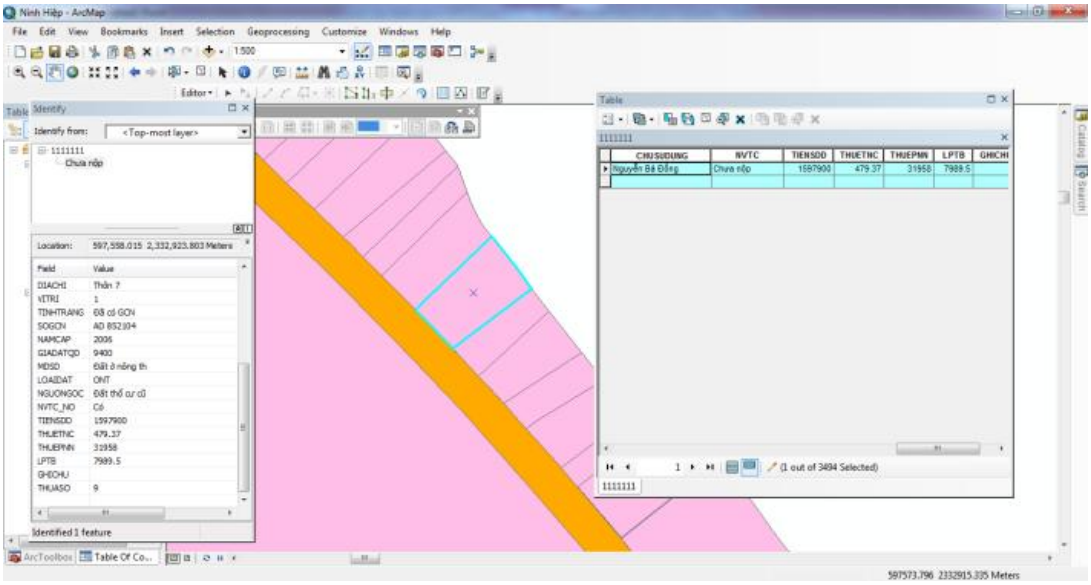

Figure 2. Land plot No. 9 on Map No. 1

- Calculation of annual tax of land use

*Tax of non-agricultural land (VND) = taxable price (VND) * tax rate (%),*

where taxable price (VND) = land price (VND/ m2) * land area (m2)

To calculate non-agricultural land use tax that must be paid by an individual household for each parcel of land, we simply created a field "ThuePNN" and use the calculation tool in ArcGIS to retrieve data from related fields and then set up a calculation formula. The tax is then determined, for instance, households with unpaid lanuse tax (Figure 3) or financial obligation on land transfer (Figure 4).

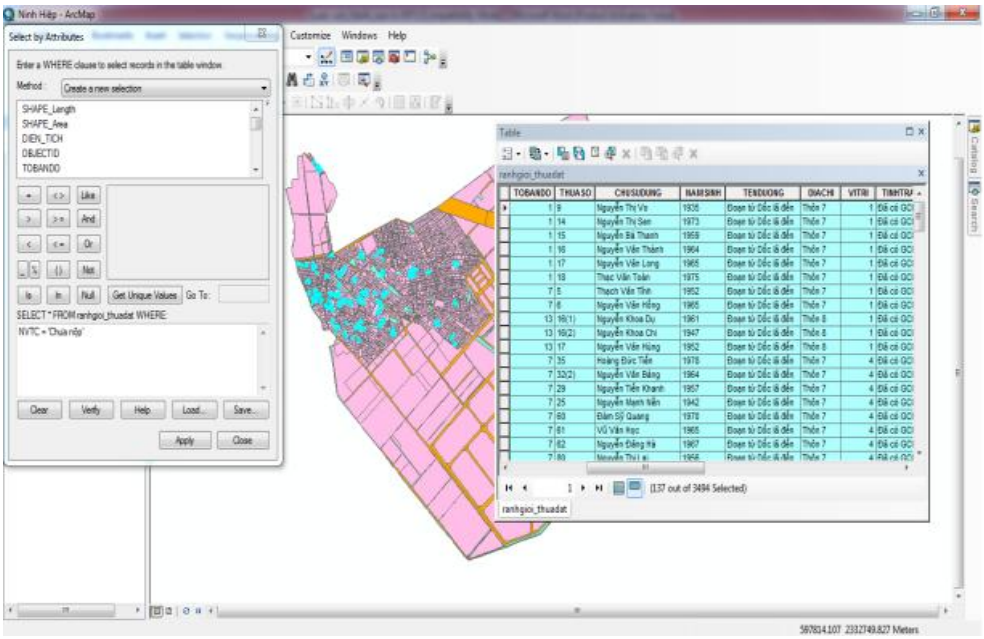

Figure 3. Households owing LU tax

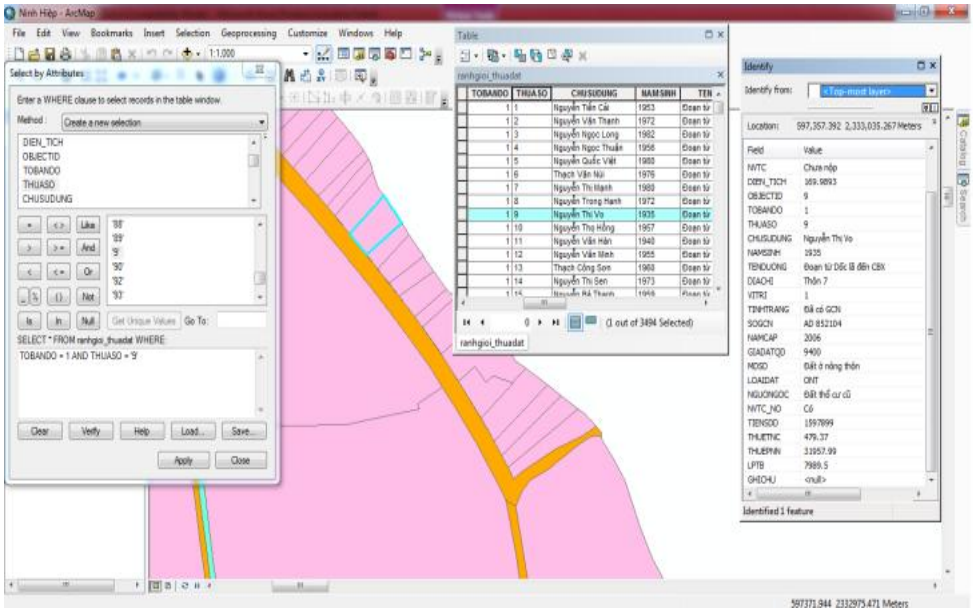

Figure 4. Financial obligation on land transfer from Mrs. Nguyen Thi Vo to Mr. Nguyen Ba Dong

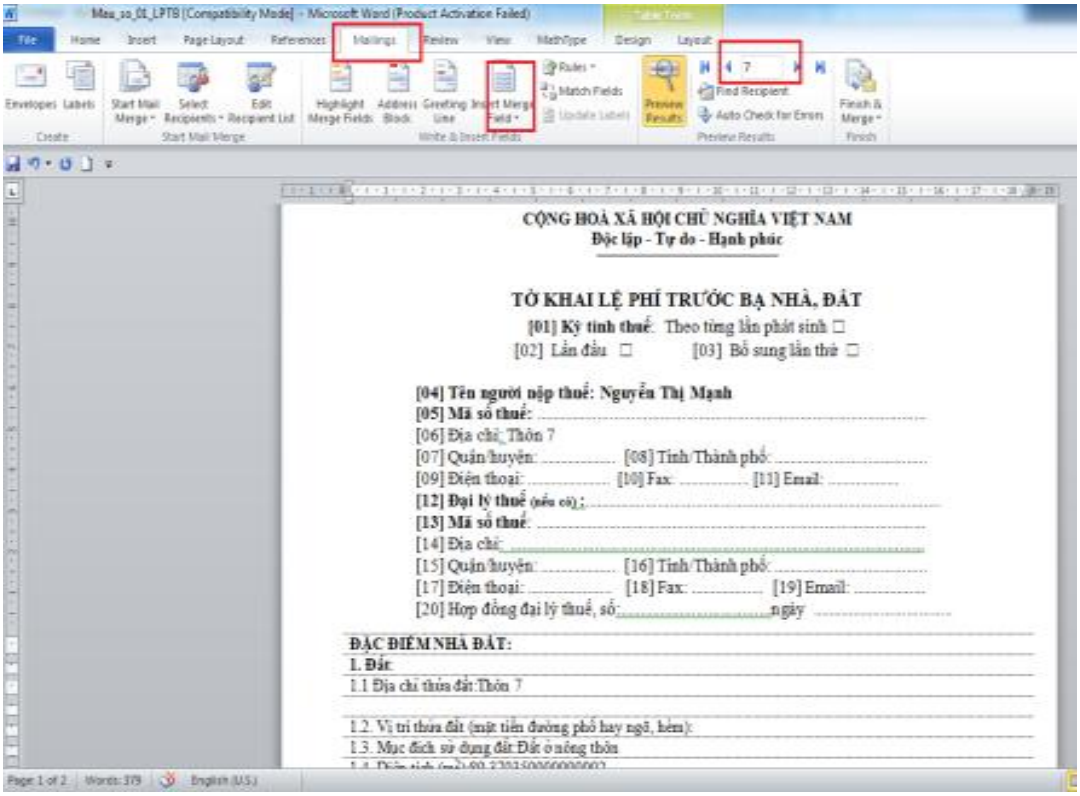

Figure 5. Result of land transferring fee declaration

3.3.4. Support procedures for declaration of financial obligations on land

With the complete database system created after the above mentioned database building process, using the Mail Merge feature of Microsoft Word with a few simple operations, a complete documentation set for people can be generated. This contributes to a reform of administrative procedures and to improving the efficiency of the service of the natural resources and environmental management.

3.3.5. Land price mapping

A land price map of Ninhhiep Commune was produced and colors were used to represent different land price ranges.

The highest land price is 9.4 million VND/m^2 at the location No 1 from La Slope to Canh Buom Xanh Eco Area

The lowest land price is 4.54 million VND/m^2 at location No 4 from Canh Buom Xanh Ecotourism site to the end of Ninh Hiep commune.

Average land price in Ninh Hiep commune is about VND 6.97 million per square meter.

3.3.6. Other functions of the database system

In addition to the above described applications, the database also enables land managers to perform business tasks quickly, such as calculation of the area of land loss when performing land clearance (Figure 6) and calculation of compensation for households (Figure 7). Data managers are also more likely to promptly update land changes as well as financial information on land to ensure that the land database is always accurate.

3.4. Sharing database on WebGIS

3.4.1. Sharing database on ArcGIS Online

To share the database on WebGIS, an account of ArcGIS Online was created and maps generated from ArcGIS Desktop were shared on ArcGIS Online. From here, spatial data and attribute data are managed and stored in the My Content section of ArcGIS Online

(Figure 8). Data on the Web is secured by specific access permission.

3.4.2. Developing a website which provides database for Ninhhiep commune

From the database that has been shared on ArcGIS Online, users build their own websites for managing and manipulating data through the preconfigured ArcGIS Online functions [2]. This also helps users to manage the database more scientifically and to create data links with other websites (Figure 9).

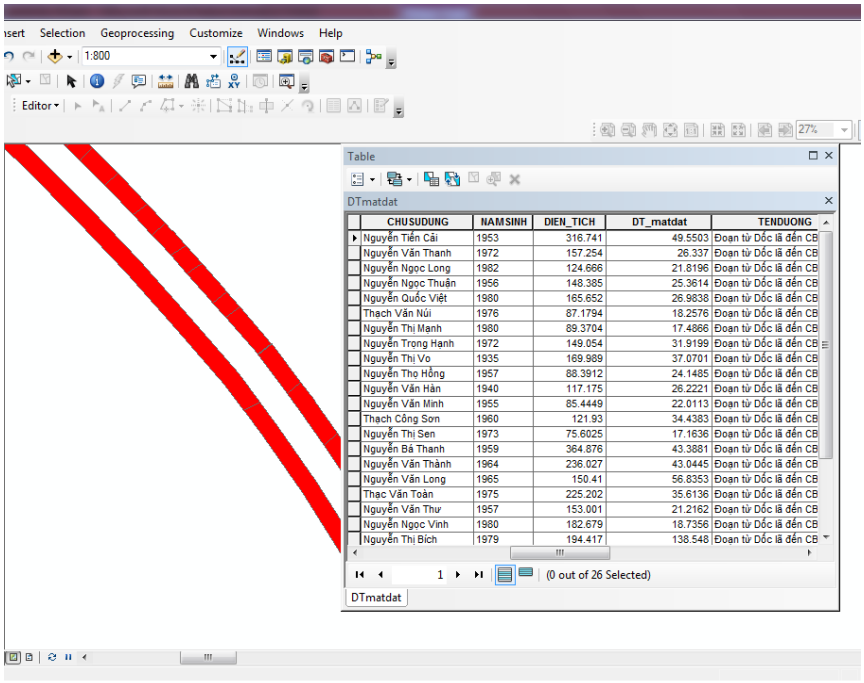

Figure 6. The area of land loss when performing land clearance

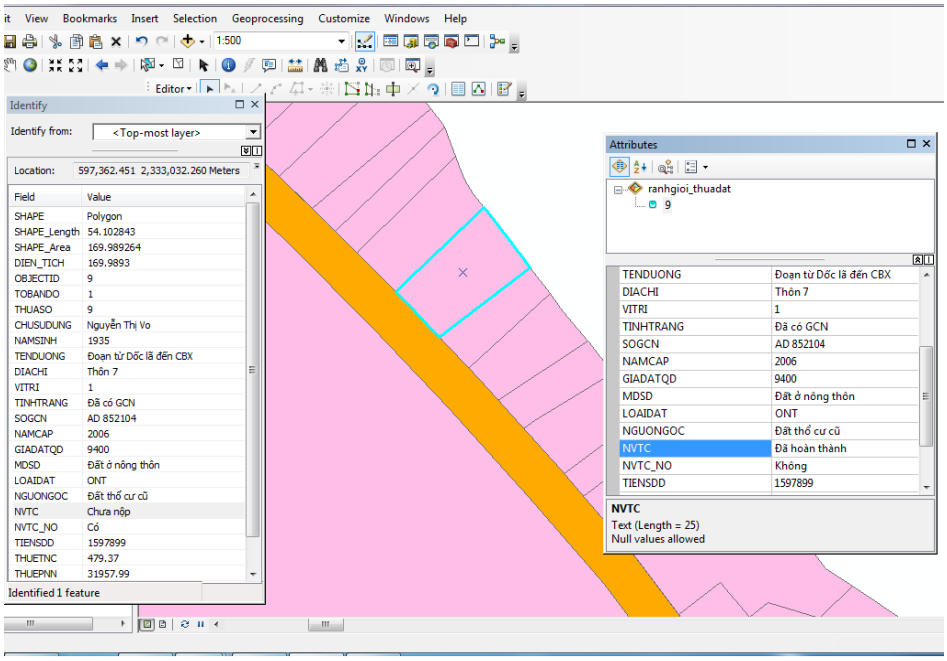

Figure 7. Update financial information on land plot No. 16

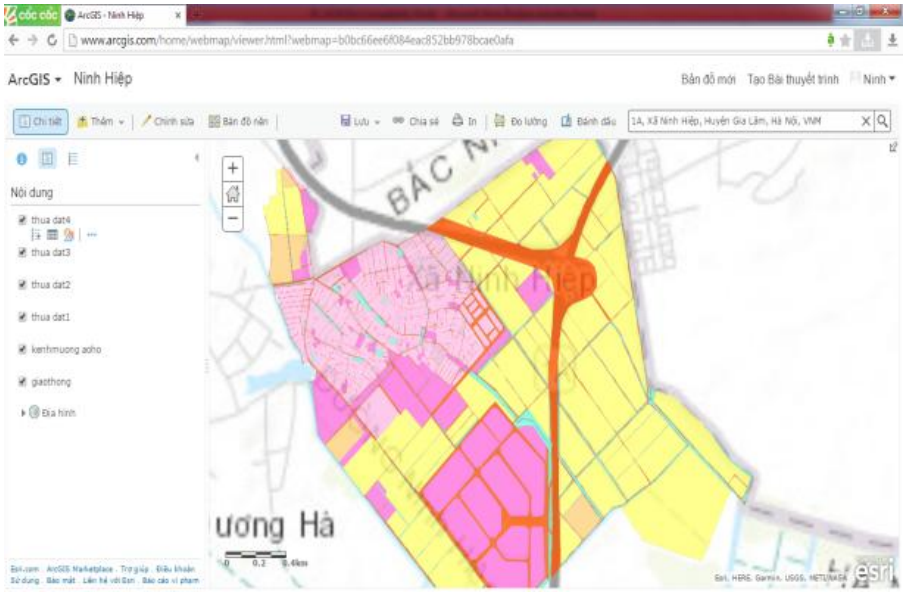

Figure 8. Data on land price is stored on My Content section

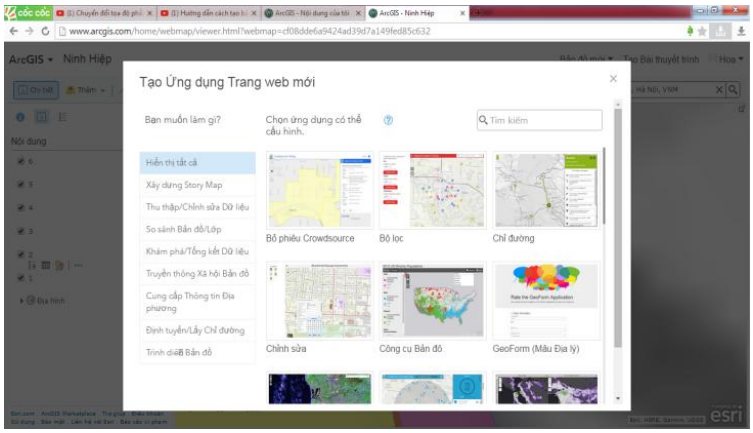

Figure 9. Create application on website

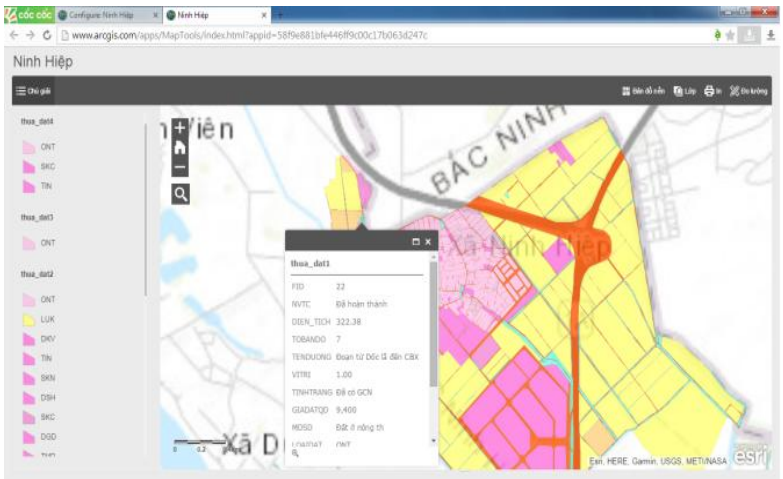

Figure 10. Image of main Website with Cadastral database of Ninhhiep commune

After completing the setup of necessary items in the website, the process of building a simple WebGIS database based on ArcGIS Online technology has been completed. Figure 12 shows the interface of the website. Shortcut keys were displayed on the website interface to help users find information, classify information layers, and retrieve information. Data administrators can update information, modify, edit, print, and create maps for presentations directly on the web, and then export them to Microsoft PowerPoint for presentation when needed.

It has been proven from research and application that WebGIS is a modern technology with many advantages and having ability to bring high efficiency to the management of land information, helping implement issues related to land allocation, land lease, land use right transfer, and land use purpose conversion. This is the basis and foundation for the establishment of legal relationships between the state and land users. A number of studies on the applications of WebGIS in land information management have been conducted (Vo Quoc Anh *et al.*, 2014; Vu Hoang Thuong, 2015). It should be noted that the input database system should be standardized based on thematic layers of land information. This is the most important stage in the process of creating the precision of handling, integration and spatial analysis of data to produce the output. The research subject concerns individual land parcels so that the motto of "grasp firmly, manage tightly" the current situation and changes of land use can be achieved. From that, managers can catch up with land developments and make appropriate and sound decisions.

4. CONCLUSIONS

The database for Ninhhiep commune was completely and synchronously constructed, detailing each parcel of land. This database includes spatial data (land parcel maps with 3494 parcels) and attribute data with 18 fields describing land users, land parcels, land prices, and legal status of land parcels. The database for Ninhhiep commune could be used to serve a number of tasks in financial management of land in the commune such as defining financial obligations of land users, summing up status of financial obligation fulfillment, calculating annual land use tax as well as other financial obligations when land users perform their rights, creating a map of land price and some other relevant applications during the implementation of land management at grass-root level.

During the course of use, when there is a change in the status of land parcels, both in terms of morphology and properties, the database can be quickly updated.

Database on the Website is stored and secured on ESRI cloud computing technology to avoid attacks from malicious programs. However, in order to access the ArcGIS Online application, an account provided by ESRI is required. ArcGIS Online allows the use of a non-commercial account during a 60-day trial period so many of its functions could not be fully exploited. To use the database effectively for financial management of land in the study area, investment on material facilities and personnel training from the goverment are strongly recommended.

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Vietnam J. Agri. Sci. 2017, Vol. 15, No. 9: 1205 - 1212 Tạp chí Khoa học Nông nghiệp Việt Nam 2017, 15(9): 1105 - 1212
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SCREENING AND CHARACTERIZATION OF CELLULASES PRODUCED BY *Bacillus* spp.

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Received date: 04.08.2017 Accepted date: 18.09.2017

ABSTRACT

Cellulases are enzymes synthesized by diverse groups of microorganisms. These enzymes have demonstrated biotechnological potentials in various sectors, including food, animal feed, brewing and wine, and pulp and paper industries. In this study, 100 strains of *Bacillus* spp. were incubated in MT1 agar medium, and cellulolytic activity was qualitatively determined by measuring the diameters of the hydrolytic clear zones. The three most active strains (A1.2, A1.8, and B6.4) showing clear zone diameters above 24 mm were selected. Cellulase activity in a liquid medium was further quantitatively determined by the dinitrosalicylic acid (DNS) method. Cellulolytic bacteria were identified by 16S rRNA gene sequencing, and neighbor-joining phylogenetic analysis was conducted to determine the evolutionary relationships between the selected strains and reported strains from the GenBank database. The strains were identified as *Bacillus cereus* (A1.2 and A1.8), and *Bacillus pumilus* (B6.4). Cellulase produced by *B. pumillus* B6.4, a GRAS bacterium showing the highest cellulase production in a liquid medium, was partially purified and characterized. The enzyme was most active at 55°C and pH 6.5. Half-lives (conducted at pH 5.0) of the enzyme at 55, 65, 75, and 85°C were 180, 180, 30 and 20 min, respectively. Similarly, half-lives (conducted at 37°C) of the enzyme at pH 5.5, 6.5, 7.5, and 8.5 were 130, 135, 80 and 70 min, respectively. The broad range of working temperatures and the stability under mild acidic conditions suggest that the cellulase of *B. pumilus* B6.4 could be a good candidate for application in the lignocellulosic bioethanol industry.

Keywords: *Bacillus* pumilus B6.4, cellulase, enzyme characterization.

Sàng lọc vi khuẩn *Bacillus* sp. sinh cellulase và xác định đặc tính của enzyme

TÓM TẮT

Cellulase là enzyme được sinh ra từ nhiều loại vi sinh vật khác nhau. Tiềm năng sinh học của những enzyme này thể hiện bởi sự ứng dụng đa dạng của nó trong các ngành công nghiệp khác nhau như thực phẩm, thức ăn chăn nuôi, bia và rượu vang, bột giấy và giấy. Tong nghiên cứu này, 100 chủng *Bacillus* sp. được ủ trong môi trường MT1 để xác định khả năng thủy phân cellulose thông qua đo đường kính vòng phân giải. Ba chủng (A1.2, A1.8 và B6.4) có đường kính vòng phân giải lớn nhất 24 mm được lựa chọn cho nghiên cứu tiếp theo. Hoạt độ cellulase được xác định gián tiếp thông qua định lượng đường khử bằng phương pháp acid dinitro-salicylic (DNS). Tên loài vi khuẩn sinh cellulase cao được xác định bằng cách giải trình tự gen 16S rRNA và sử dụng cây tiến hóa để hiển thị mối quan hệ giữa các chủng được chọn với các chủng khác trong cơ sở dữ liệu. Các chủng này được xác định là *Bacillus cereus* (A1.2 và A1.8) và *Bacillus pumilus* (B6.4). Cellulase tạo ra từ *Bacillus pumilus* B6.4, một vi khuẩn được xếp vào nhóm an toàn (GRAS), cho kết quả là cao nhất khi nuôi cấy trong môi trường lỏng sẽ bước đầu được tinh sạch và xác định đặc điểm. Enzyme cellulase hoạt động tốt nhất ở 55°C với pH 6.5. Thời gian bán rã (tiến hành ở pH 5.0) của enzyme ở nhiệt độ 55, 65, 75 và 85°C tương ứng là 180, 180, 30 và 20 phút. Tương tự, thời gian bán rã (tiến hành ở 37°C) của enzyme ở pH 5,5, 6,5, 7,5 và 8,5 lần lượt là là 130, 135, 80 và 70 phút. Phạm vi rộng của nhiệt độ tác động và sự ổn định trong điều kiện acid nhẹ cho thấy cellulase của *Bacillus pumilus* B6.4 có thể là ứng cử viên tốt sử dụng trong ngành công nghiệp nhiên liệu sinh học

Từ khóa: *Bacillus pumilus* B6.4, cellulase, đặc tính enzyme

1. INTRODUCTION

Cellulases contribute to 8% of the worldwide industrial enzyme load and demand is expected to increase drastically in the near future (Costa *et al.*, 2008). Cellulase is significant due to its key roles in biotechnology and industrial applications (Bhat, 2000). It has been widely utilized for bioremediation (Zahangir *et al.*, 2005), food processing (Chandara *et al.*, 2005), paper and pulp industry, supplementation in the animal feed industry (Chandara *et al.*, 2005), textile industry (Ali and Saad, 2008), alcoholic beverage, malting, and brewing (Sreeja *et al.*, 2013), formulation of washing powders, extraction of fruit and vegetable juices, and starch processing (Camassola and Dillon, 2007).

Although both fungi and bacteria have been exploited for their abilities to produce a wide variety of cellulases and hemicellulases, the isolation and characterization of novel cellulases from bacteria have become increasingly intensive. There are several reasons for these movements: i) bacteria often have a higher growth rate than fungi allowing for higher recombinant production of enzymes, ii) bacterial cellulases are often more complex and are in multi-enzyme complexes providing increased function and synergy, and iii) bacteria inhabit a wide variety of environmental and industrial niches like thermophilic or psychrophilic, alkaliphilic or acidophilic, and halophilic strains, which produce cellulolytic strains that are extremely resistant to environmental stresses (Sangrila and Tushar, 2013).

The aim of this study was to find novel cellulases of application potential for the cellulosic bioethanol industry from *Bacillus* sp. strains isolated in Vietnam.

2. MATERIALS AND METHODS

2.1. Materials

Bacteria strains: One hundred strains of Bacillus spp. were supplied by the Faculty of Food Science and Technology, Vietnam National University of Agriculture. These strains were collected from two different sources, namely from chili sauce (Muong Khuong, Lao Cai, Vietnam), and from cow rumen (Bavi, Hanoi, Vietnam).

2.2. Methods

The experimental flow diagram for the screening and characterization of cellulases produced by *Bacillus* spp. is indicated in Figure 1.

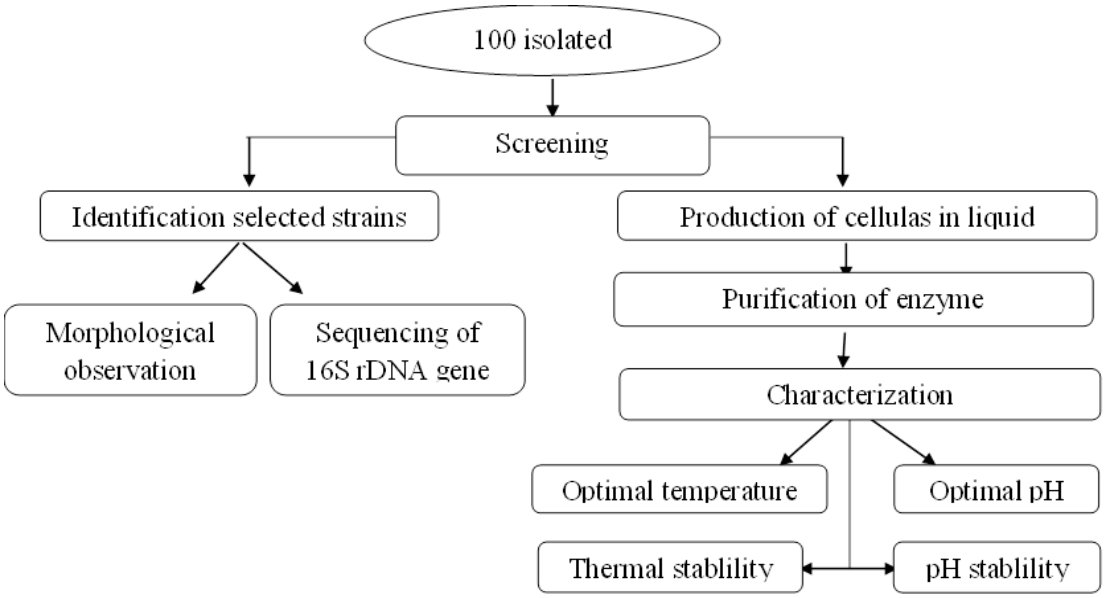

Fig. 1. Screening and characterization of cellulases produced by *Bacillus* spp.

- Screening of cellulase producing bacteria

Microorganisms were activated in NB medium (per liter: 10 g peptones, 5 g NaCl, 5 g meat extract, pH 7), and then 100 µl of culture broth was sporred on an agar plate containing MT2 medium (per liter: 20 g CMC; 0.25 g yeast extract; 3.5 g KH_2PO_4; 5 g $MgSO_4$; 0.625 g KNO_3; 20 g agar; pH 7) (Thi and Quyen, 2014) for primary detection of cellulase. The formation of a clear zone of hydrolysis indicated cellulose degradation. The difference (in mm) of the clear zone and colony diameters qualitatively reflecting enzyme activity was recorded. Enzyme activity was also determined by using the 3,5-dinitrosalicylic acid (DNS) method (Miller *et al.*, 1959). The strains showing the highest cellulase activity were selected for further study.

- Cellulase enzyme assay

Briefly, the cellulase activity (U/ml) was measured by estimation of reducing sugars liberated from CMC. A 1% CMC solution was prepared in 50 mM sodium acetate buffer (pH 5.0). The enzyme assay was performed by incubating 0.1 ml enzyme with 0.9 ml of 1% CMC solution at 37°C for 30 min. After incubation, the reaction was stopped by the addition of 1.2 ml of DNS reagent, and boiled at 100˚C in a water bath for 10 min. Liberated sugars were determined by measuring the absorbance at 540 nm. Cellulase production was estimated by using a glucose calibration curve. One unit (U) of enzyme activity was expressed as the quantity of enzyme required to release 1 µmol of reducing sugar per minute under testing conditions (37°C, pH 5.0) (Singh *et al.,* 2013).

- Identification of selected strains

The strains showing the highest cellulase activities were identified based on morpho-physiological characteristics (Gram staining, colony and cell morphology, mobility) (Apun *et al.*, 2000) and 16S rRNA gene sequencing.

- Analysis of 16S rRNA gene sequence

Genomic DNA was extracted and purified using CTAB (Current Protocol in Molecular Biology, 2009), and DNA purity was spectrophotometrically assessed by the A260/A280 ratio. The fragment of the 16S rRNA gene was amplified using the universal primers 8F (5 -AGAGTTTGATCCTGGCTCAG-3') and 1492R (5′-GGTTACCTTGTTACGACTT-3'). The PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 90 sec; 72°C for 5 min. A positive control (*E. coli* genomic DNA) and a negative control (without DNA template) were also used in the PCR reactions. DNA products (about 1.5 kb) were gel purified using a QIAquick Gel Extraction Kit (Qiagen) as recommended by the manufacturer and sent out for sequencing (First BASE Laboratories, Selangor, Malaysia). DNA sequences were then compared to published sequences in GenBank using the BLAST engine hosted at NCBI (Bethesda, USA).

- Characterization of cellulase

For the crude enzyme preparation, a fresh colony of a selected strain was inoculated in 2.5 ml of NB medium at 37°C on a rotary shaker at 150 rpm for 24 h. After that, 2 ml of culture broth was added into 250 ml of MT1 medium (per liter: 10 g CMC; 1g D-glucose; 2 g yeast extract; 0.5 g KH_2PO_4; 0.2 g $MgSO_4.7H_2O$; 0.04 g $CaCl_2$; 0.02 g $FeSO_4.7H_2O$; 0.75 g KNO_3; pH 7) (Lisdiyanti *et al.,* 2012) in a 500 ml conical flask and incubated at 37°C, 150 rpm for 24 h. The broth was then centrifuged at 6000 rpm for 15 min at 4°C and the supernatant was used as crude enzyme for further studies.

Partial purification of cellulase: Cellulase was precipitated by adding 4 volumes of 96% ethanol. The slurry was centrifuged at 6000 rpm, 4°C for 30 min, and the supernatant was discarded. The pellet was washed three times with 50 mM phosphate buffer, pH 7.0, and then re-dissolved in 20 ml of sodium acetate buffer, pH 5.0. The partially purified enzyme was used for further characterization.

Optimum temperature and pH: For determination of the optimum temperature, reaction mixtures containing the enzyme preparation and 1% CMC in 50 mM sodium acetate buffer, pH 5.0 were incubated at different temperatures, ranging from 40°C to 80°C for 30

min. Relative cellulolytic activity was determined by measuring the amounts of reducing sugar released. Similarly, optimum pH was determined by incubating the reaction mixtures at different pHs, ranging from 5.0 to 8.0 at 37°C using 50 mM sodium acetate buffers.

Temperature and pH stability: Thermal stability was investigated by pre-incubating the partially purified enzyme at various temperatures, ranging from 45°C to 85°C. At different time points, samples were taken and residual cellulase activity was determined by a reducing sugar assay using the DNS method. The relative activity at different time points was calculated as a percentage of the maximum activity observed for each given temperature. Similarly, pH stability was studied by pre-incubating the enzyme at 37°C in 50 mM sodium acetate buffer with the pH ranging from 5.5 to 8.5. The residual cellulase activity was determined at different time points as described above.

3. RESULTS AND DISCUSSION

3.1. Screening of cellulase producing bacteria

All 100 *Bacillus* spp. strains were cultured on CMC agar plates for screening of cellulase activity. The diameters of clear zones produced by the tested strains varied from 3 to 24 mm. The results are summarized in Table 1. Among the tested stains, we eliminated 32 strains since they were morphologically identical and produced clear zones with diameters less than 10 mm. There were 9 isolates that showed large clear zones (21-25 mm).

Although it indicates hydrolytic activity, the plate-screening method is not quantitative and there is a poor correlation between enzyme activity and the size of the clear zone (Maki *et al.,* 2009). Thus, the three strains (A1.2, A1.8, B6.4) that showed largest clear zones (≥ 24 mm) were selected for further screening on the basis of CMCase production in a liquid medium.

The strains were cultivated in MT1 medium containing 1% CMC at 37°C for 36 h for cellulase production. CMCase activities of the strains are presented in Table 2.

Table 2. Cellulase activity of 3 selected strains

Strain	Cellulase activity (IU/ml)
A1.2	1.14 ± 0.01
A1.8	1.01 ± 0.01
B6.4	3.01 ± 0.02

The most active cellulase producer was B6.4, which showed CMCase activity of 3.01 U/ml. It was previously reported that the maximum cellullase activity of *Bacillus velesensis* was 0.02 U/ml (Ancharida *et al.,* 2014), and *B. safensis* was 0.23 U/ml (Khianngam *et al.,* 2014). Thus, in comparison with published data, the cellulase activity of B6.4 was significantly higher.

3.2. Identification of selected strains

All 3 isolates (A1.2, A1.8, and B6.4) were Gram positive, endospore forming, and mobile. The 16S rRNA gene sequences obtained for the three selected strains were compared with the GenBank nucleotide database using the BLAST tool. Strains A1.8 and A1.2 were most closely related to *Bacillus cereus* and shared 99% and 97% homology, respectively, to the latter. Strain B6.4 was most closely related to *Bacillus pumillus* YQQ24 (97% of homology). The phylogenetic tree generated using the 16S rRNA gene sequences for *B. pumilus* B6.4 is presented in Figure 2. According to the FDA (2015), *B. pumilus* is regarded as GRAS. *B. pumilus* B6.4 was therefore chosen for further studies.

3.3. Characterization of cellulase produced by B. pumilus B6.4

- Optimum temperature: Temperature greatly affects enzyme activity. The effect of temperature on *B. pumilus* B6.4 cellulase was studied in the range from 40°C to 80°C with 5°C intervals. The results revealed that cellulase activity of *B. pumilus* B6.4 increased when the temperature increased from 40°C to 50°C, and reached a maximum at 55°C (5.157 U/ml), then activity gradually decreased as the temperature increased to 80°C (Figure 3).

Table 1. Clear zones produced by 100 tested *Bacillus* spp. strains on CMC agar

Diameter of clear zone (mm)	Number of strains	Source of isolation	
		Chili sauce (A)	Cow rumen (B)
0-5	5	2	3
6-10	27	7	20
11-15	34	11	23
16-20	25	6	19
21-25	9	2 (A1.2; A1.8)	7 (B2.6, B2.7, B4.6, B4.9, B5.5, B6.4, B8.1)

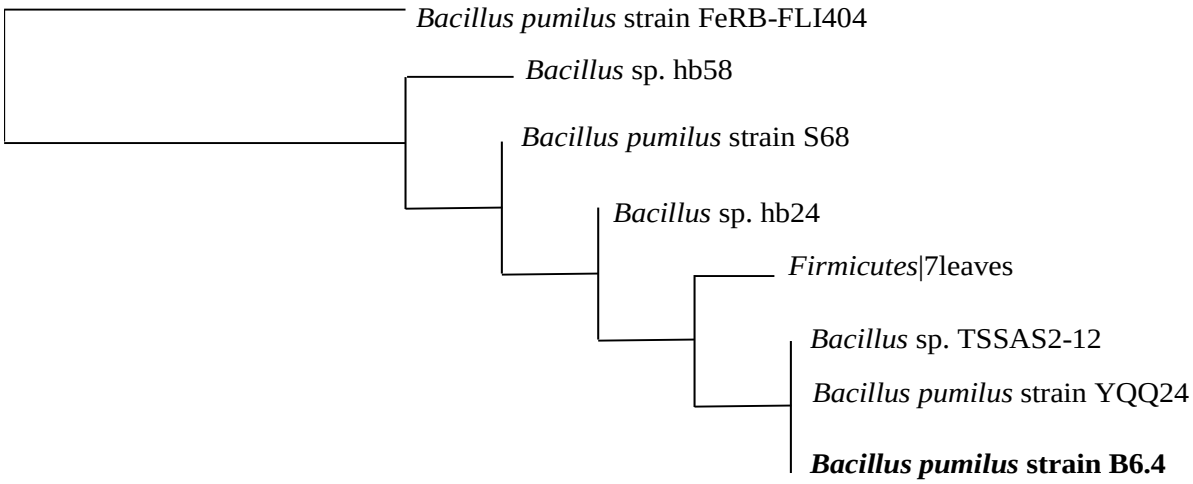

Fig. 2. Phylogenetic tree based on the 16S rDNA sequences of *B. pumilus* B6.4

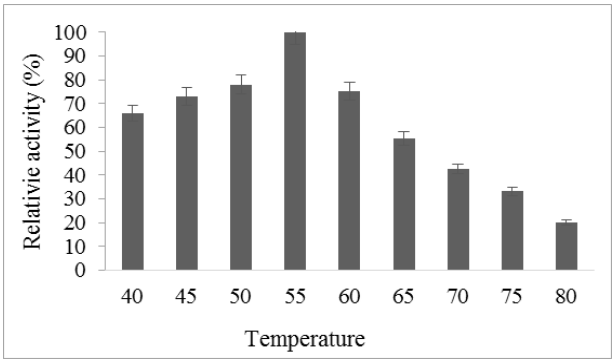

Fig. 3. The effect of temperature on *B. pumilus* B6.4 cellulase activity

The optimum temperature (55°C) of *B. pumilus* B6.4 cellulase was slightly different from that previously reported for *B. pumilus*. *B. pumilus* S124A cellulase functioned optimally at 50°C (Natesan and Nelson, 2014), and 60°C was the optimum temperature for *B. pumilus* EB3 cellulase (Ariffin *et al.,* 2006). Other *Bacillus* cellulases also share a similar range of optimum temperatures, for example, cellulases of *B. mycoides* S122C and *Bacillus subtilis* YJ1 have optimum temperatures of 50°C (Balansubramanian *et al.*, 2012), and 60°C (Li *et al.,* 2010), respectively.

- pH optimum: *B. pumilus* B6.4 cellulase showed the highest activity at pH 6.5, but it also demonstrated rather high activity in light

acidic (pH 5.5 - 6.0) and mild alkaline (pH 7.5) conditions (Fig. 4). For instance, at pH 5.5 and pH 7.5, the enzyme retained 79% and 80% of its maximum activity, respectively. However, a steep reduction in cellulase activity was noticed in alkaline conditions. At pH 8, the relative activity was only 36%.

Optimum pH values of 4.5 to 8.0 have been reported for different microbial cellulases (Immanuel *et al.,* 2007; Dutta *et al.,* 2008). Each enzyme has its own optimum pH and if the pH increases or decreases beyond the optimum, the ionization groups at the active site may change by slowing or preventing the formation of an enzyme substrate complex (Eijsink *et al.,* 2005). The pH range suitable for cellulase from *B. circulans* was found to be 4.5 to 7.0 (Kim, 1995). For other *Bacillus* strains, pH optima were 5.0 to 6.5 (Mawadza *et al.,* 2000) and 6.0 to 6.5 in *B. subtilis* YJ1 (Li *et al.,* 2010).

- Thermal stability

Thermal stability of cellulase from *B. pumilus* B6.4 was determined by measuring the relative cellulolytic activity at various temperatures, ranging from 45°C to 85°C, and at different time points, from 30 to 240 min at pH 5.0 (Fig. 5). More than 58% of cellulase activity was maintained at temperatures ranging from 55 to 65ºC after 150 min incubation at pH 5.0 and it remained more than 40% after 240 min. Interestingly, the enzyme was less stable at 45°C. At 45ºC, about 38% of the activity was maintained after 150 min and then dramatically decreased to 25% after 240 min. Cellulase degradation at 45°C could be explained by the presence of associated proteases, although this might require further verification. At 75°C and 85°C, less than 15% of the activity was observed after 150 min, and 1% after 240 min.

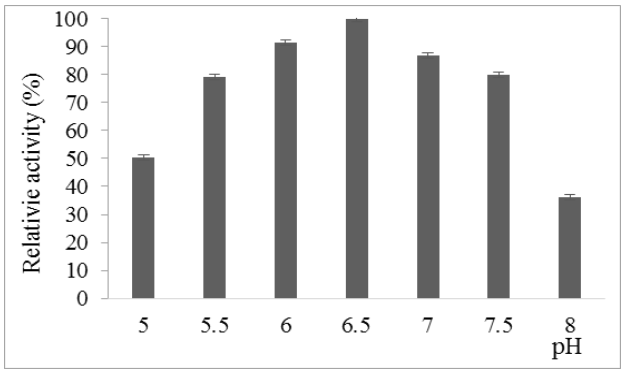

Fig. 4. The effect of pH on *B. pumilus* B6.4 cellulase activity

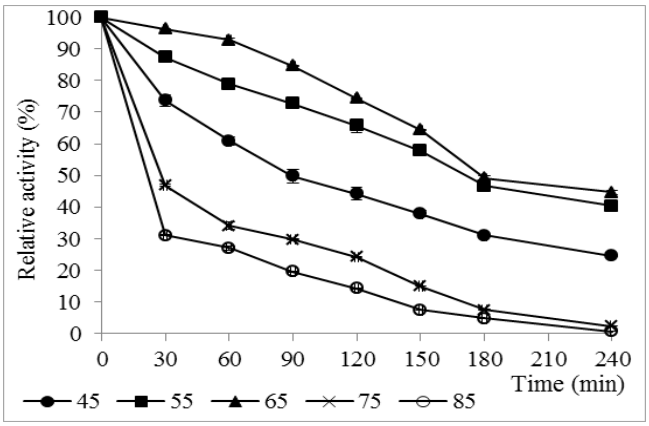

Fig. 5. Thermal stability of *B. pumilus* B6.4 cellulase

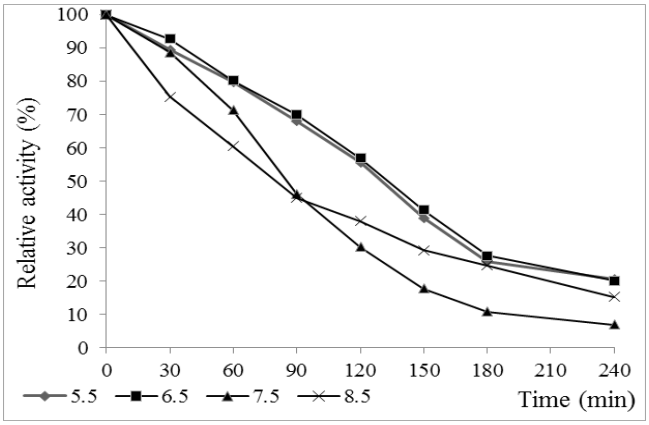

Fig. 6. pH stability of *B. pumilus* B6.4 cellulase

Natesan and Nelson (2014) reported that *B. pumilus* S124A cellulase was stable at 60°C to 70°C. Cellulase from *B. amyoliquefaciens* DL-3 was stable at temperatures ranging from 50°C to 70°C (Lee *et al.,* 2008), and purified cellulase produced by *B. subtilis* was stable at 40°C to 60°C (Rekha and Lakshmi, 2012). Cellulases produced by *Bacillus* sp., *Pseudomonas* sp., and *Serratia* sp. isolates were found to be stable up to 55°C (Prabesh *et al.*, 2016).

- pH stability

The stability of the enzyme when incubated at different pHs between 5.5 and 8.5 was determined (Fig. 6). The results showed that more than 56% of cellulase activity was maintained at a pH range of 5.5 to 6.5 after 120 min of incubation, and more than 21% after 240 min. About 30% of activity remained at pH 7.5 to 8.5 after 120 min and then dramatically decreased to 7% after 240 min.

Thus, cellulase obtained from *B. pumilus* B6.4 was rather stable at a pH of 5.5 to 6.5. Some previous studies have also reported that cellulases produced by several *Bacillus* sp. were stable over a wide pH range (Mawadza *et al.,* 2000; Lee *et al.,* 2008). *B. subtilis* BY-4 cellulase was found to be stable at a pH ranging from 4.5 to 6.0, and most stable at pH 5.0 (Lima *et al.,* 2015). *B. amyloliquefaciens* DL-3 cellulase was stable over a broad pH range, from 4.0 to 9.0 (Lee *et al.,* 2008), *B. halodurans* IND18 cellulase showed stability at a pH from 6.0 to pH 9.0, and was most stable at pH 9.0 (Gao *et al.,*2008).

4. CONCLUSIONS

One hundred strains of *Bacillus* spp. were qualitatively screened for cellulase activity using an agar plate assay and the three most active strains (A1.2, A1.8, and B6.4) were selected. Strains A1.2 and A1.8 were identified as *Bacillus cereus,* and B6.4 as *Bacillus pumillus* based on 16S rRNA gene sequencing. Cellulase produced by *B. pumillus* B6.4, a GRAS bacterium showing the highest cellulase production in a liquid medium, was partially purified. The cellulase was most active at 55°C and pH 6.5. The enzyme maintained more than 58% activity after treatment at 55°C or 65°C for 150 min. It also maintained more than 56% activity at pH 5.5 or 6.5 for 120 min. These properties suggest that *B. pumilus* B6.4 could be a good candidate for application in the cellulosic biofuel industry.

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ISOLATION, SELECTION AND IDENTIFICATION OF *Aspergilus oryzae* FROM TRADITIONAL FERMENTED FOODS PRODUCING HIGH SALT TOLERANT NEUTRAL PROTEASE

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Received date: 14.08.2017 Accepted date: 18.09.2017

ABSTRACT

This study aimed to isolate, select, and identify *Aspergillus oryzae* producing high salt tolerant neutral protease from traditional fermented foods which could potentially be used for food fermentation and other relevant applications under the high salt conditions. Twelve of 23 isolates were primarily assumed to be *Aspergillus oryae* by morphological observation. Of which, TB1 from soy sauce revealed the highest protease activity with 49.26 U/l, corresponding to a 17mm diameter clear zone on a BCG casein agar plate, and was identified using molecular biology methods and named *Aspergillus oryzae* TB1. Protease activity of this strain was highly active in the pH range of 5.0 - 8.0 and was optimal at pH 7.0. The enzyme activity remained 70% after 8 hours of incubation at pH 7.0 and 37^{o}C.The protease activity of TB1 was reduced when the sodium chloride concentration increased from 0% to 16%, and relative activity was 51.8% at 16% NaCl. In the salt tolerance test, the results indicated that the protease tolerated 16% NaCl and residual activity was 49.2% after 9 hours of incubation at 37°C.

Keywords: *Aspergillus oryzae*, protease activity, salt tolerance

Phân lập, tuyển chọn và định danh *Aspergillus oryzae* có khả năng sinh protease trung tính và chịu mặn cao từ một số thực phẩm lên men truyền thống

TÓM TẮT

Nghiên cứu này nhằm phân lập, tuyển chọn và định tên *Aspergillus oryzae* từ một số thực phẩm lên men truyền thống có khả năng sinh protease trung tính và chịu mặn cao, có tiềm năng ứng dụng trong lên men thực phẩm và các ứng dụng khác ở điều kiện muối cao. Mười hai trong 23 chủng được định danh sơ bộ là *Aspergillus oryzae* bằng phương pháp quan sát hình thái. Trong đó, chủng TB1 phân lập từ tương bần sinh protease cao nhất với 49,26 U/l, tương ứng với đường kính 17mm của vòng phân giải trên đĩa thạch chứa BCG được định danh bằng phương pháp sinh học phân tử và đặt tên là *Aspergillus oryzae* TB1. Protease của chủng này có hoạt độ cao trong khoảng pH 5,0 - 8,0 và tối ưu ở pH 7,0. Hoạt độ enzyme vẫn còn 70% sau 8 giờ ủ ở pH 7,0 và 37^{o}C. Nhìn chung, hoạt độ protease giảm khi nồng độ muối natri clorua tăng từ 0 đến 16%, hoạt độ tương đối là 51,8% ở 16% NaCl và nồng độ muối này được sử dụng để xác định khả năng chịu mặn của protease. Kết quả cho thấy hoạt độ enzyme còn lại là 49,2% sau 9 giờ ủ ở 37^{o}C.

Từ khóa: *Aspergillus oryzae*, hoạt độ protease, chịu mặn.

1. INTRODUCTION

Proteases are multifunctional enzymes and extremely important in the pharmaceutical, medical, biotechnology, and, particularly, food industries, accounting for nearly 60% of the whole enzyme market (Ramakrishna *et al.,* 2010). Proteases are ubiquitous, however, high salt tolerant neutral proteases are receiving considerable attention as these enzymes are currently commercially limited.

Proteases can be classified into three types based on their optimal pH. Neutral proteases are more important for the food industry because

they can hydrolyze the proteins of raw materials thoroughly and reduce the bitterness. They are mainly used in the industries of food fermentation, brewing and feed additives, etc. In addition, some kinds of food are unique due to their high concentration of sodium chloride. Higher sodium chloride contents provide a lower degree of protein degradation. The salt resistant proteases are used in fermented food production, antifouling coating preparation, and waste treatment, especially in marine habitats (Gao *et al.*, 2016). The protease activity and stability are decreased sharply when the materials are mixed with sodium chloride at a high concentration, which is used for inhibiting spoilage bacteria, selectively retaining the slow growth of osmotolerant yeast and lactic acid bacteria, as well as prolonging the preservation time. Consequently, a protease that could tolerate a high concentration of sodium chloride is important in order to improve food quality, to shorten the time for the maturation process, and to improve the efficiency of raw material utilization (Wang *et al.*, 2013). In food fermentation, such as in koji or sauce production, a protease capable of high salt concentration tolerance is very necessary. A recent study by Mueda (2015) revealed that the sodium chloride content found in commercial fish sauce was 20 - 25%. It was considered as high salt product due to its 20 - 25% salt content.

Since proteases are physiologically necessary for living organisms, they are found in a wide diversity of sources including plants and animals, but commercial proteases are produced exclusively from microorganisms. Fungi of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe, of which, *Aspergillus oryzae* is of particular interest (Chutmanop *et al.*, 2008). This fungus is a potential source of proteases due to its high proteolytic activity, broad biochemical diversity, susceptibility to genetic manipulation, high productivity, and is extracellular and thus is easily recoverable from the fermentation medium (De Castro and Sato, 2014).

2. MATERIALS AND METHODS

2.1. Materials

Soy sauce, fish source, Miso sauce, Com ruou, Dau xi sauce, and fermented shrimp (Table 1) were used to isolate, select, and identify *Aspergillus oryzae* producing high salt tolerant neutral protease. The salt content in each sample was indicated by the label or by producers.

2.2. Methods

Isolation of Aspergillus oryzae from some fermented foods

The isolation was performed using the method of Nevalainen *et al.,* (2014) with several modifications. A small amount of each sample was aseptically placed onto the central part of several prepared Petri plates containing PDA medium. These plates were sealed and labeled by sample codes and incubated at 30°C. After 3 days of incubation, fungal colonies were observed growing on the surface of the medium plates, and colonies that differed in terms of appearance, size, color, morphological shape, and spread rate of mycelium were recorded.

Table 1. Fermented food samples were used to isolate *Aspergillus oryzae*

Samples	Location of samples	Symbol of sample	Salt concentration (%)
Soy sauce	Hung Yen	TB	16
Fish sauce	Hanoi	NM	25
Miso sauce	Hanoi	MS	22
Com ruou	Nam Dinh	CR	4
Dau xi sauce	Nam Dinh	DX	20
Fermented shrimp	Hue	TC	10

Morphological identification

The morphological identification of colonies as *Aspergillus oryzae* was performed by the slide culture method as described by Leck, 1999.

Identification of Aspergillus oryzae by molecular biology method

Genomic DNA was extracted using a CTAB (Cetyltrimethyl ammonium bromide) method (Doyle & Doyle, 1987).

PCR of ITS region: Two primers, ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.*, 1990), were used to amplify the complete ITS region.

Sequencing: The PCR products were purified from agarose gel using the PureLink Quick Gel Extraction Kit (Invitrogen). The mixture of purified PCR product and primer was sent to First-base, Malaysia for sequencing and reading.

Sequencing analysis: After being assembled using the SeqMan program (DNASTAR, Madison, WI), the sequences were initially compared to known ITS sequences using a BLAST search. The sequences were aligned using the ClustalX program.

Preparation of crude enzyme

Protease production followed the methods of Fernandes *et al.*, 2010 with slight modifications. After 4 days on PDA plates, 3 mycelium plugs (5 mm diameter) of actively growing fungi were taken out to inoculate PD broth medium. After 3 days of shaking incubation, the cultures were filtered and centrifuged at 4°C with 6000 rpm for 20 min. The supernatants were used for determination of enzyme activity and further characterization of the enzyme.

Determination of protease activity

Protease activity was qualitatively determined according to the well diffusion method described by Vijayaraghavan and Vincent, 2013. Agar culture was prepared along with Bromocresol green (BCG) and 1% (w/v) casein and poured in the Petri dishes. The plates were solidified for 30 min and wells (5mm diameter) were punched on each plate. One-hundred µl of crude enzyme was pipetted into each well, and then the plates were incubated at a temperature 30°C for 2 - 3 days. A zone of proteolysis was measured on the casein agar plates.

Protease activity was quantitatively assayed according to the modified method of Sigma's non-specific assay. One mL of the enzyme was incubated with 5.0 mL 0.65% casein solution at 37°C. The reaction was terminated by adding 5.0 mL trichloroacetic acid (TCA) after 10 minutes. The suspension was allowed to settle for 30 min at 37°C to precipitate the protein. The precipitate was removed by centrifuging at 6000 rpm for 10 min. Two mL of supernatant was mixed with 5 mL 0.5 M Na_2CO_3 and 1.0 mL of 1.0 M Folin reagent and incubated at 40°C for 20 min. The absorbance of the supernatant was measured with a spectrophotometer at 660 nm. One unit (U) of protease activity was defined as the amount of enzyme releasing 1.0 mmoL of tyrosine equivalent per min.

Effect of incubation time on protease activity and stability

The effect of incubation time on protease production was carried out by the method of Pant *et al.*, 2015 with slight modifications**.** The protease production was carried out in 100-mL conical flasks with agitation. Every 12 h, each flask was filtered, followed by centrifugation at 4°C and 6000 rpm for 20 min. The culture filtrates obtained were tested for protease activity.

Effect of pH on protease activity and stability

Protease activity was determined as described above but by varying the pH between 3.0 and 9.0 using Briton-Robinson buffer. To determine pH stability of the protease, enzyme samples were incubated in buffer with varied pHs at 37°C for 12 hours, and at interval times, enzyme was withdrawn to test the residual enzyme activity.

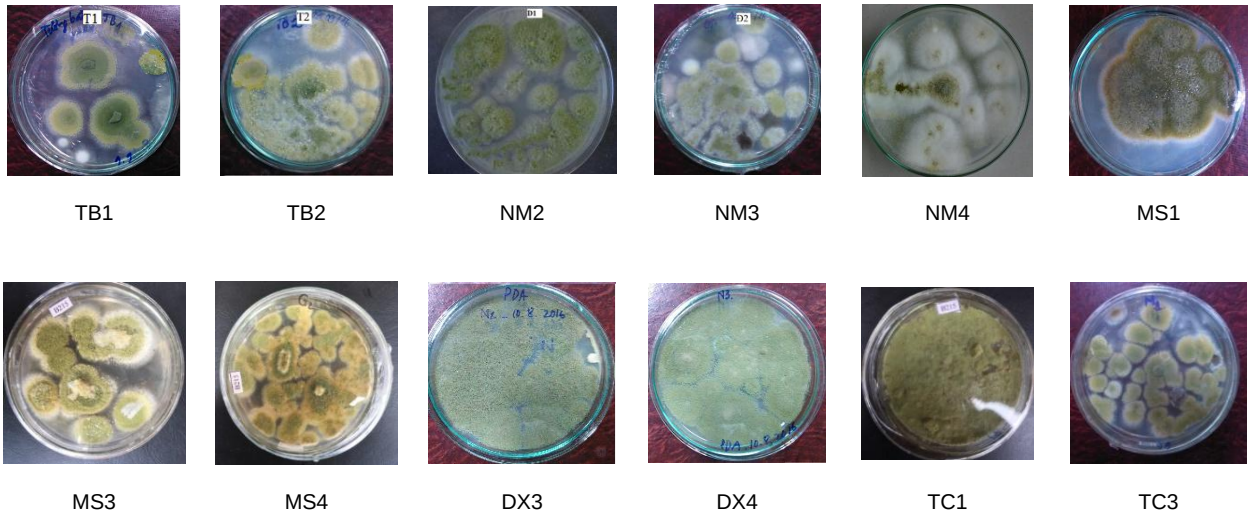


Figure 1. Isolation of *A. oryzae* from fermented foods

Effect of NaCl on the protease activity and stability

In order to study the effect of NaCl concentration, enzyme activity was measured in 50 mM potassium phosphate buffer (pH 7,5) by adding 1 mL of the crude enzyme to 1 mL of 0.65% casein in the presence of NaCl varying from 0 to 20%.

In order to determine the protease stability at a high concentration of NaCl, the crude enzyme was incubated in 16% NaCl at 30°C and pH 7.5 (phosphate buffer) for 24 hours. At interval times, enzyme was withdrawn to test the residual activity.

3. RESULTS AND DISCUSSION

3.1. Isolation of *Aspergillus oryzae* from the fermented foods

Based on colony and mycelium color, only 12 fungal colonies of 23 isolates were assumed to be *Aspergillus oryzae*. The isolates with the codes in Figure 1 were used to test protease activity.

3.2. Determination of protease activity produced from the isolates

The casein agar plate assay allows principally for qualitative determination of protease activity. The hydrolysis zones on the casein agar could be related to the amount of protease produced by the fungus (Vermelho *et al.*, 1996). After 3 days of incubation at 30°C, the clear zones were observed (Figure 2) and quantification of the protease activity was completed as indicated in Table 2. The clear distinct zones were observed after the addition of BCG reagent on the casein agar plates. The zones were distinct and the surrounding areas were greenish blue in color, but the color of the plates strongly depended on the pH value of the agar medium. In this study, the plates appeared blue in color due to the pH of the culture medium being maintained at 8.0. The TB1 isolate with highest diameter of the clear zone and enzyme activity was selected for further study.

3.3. Molecular identification of the TB1 fungus

From the qualitative protease activity test, TB1 showed the highest enzyme activity and was selected for identification using molecular biology methods. The ITS (Internal Transcribed Spacer) region of the TB1 strain was used for identification. Two primers, ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC 3') were used to amplify the complete ITS region of TB1. The expected PCR product, around 700 bp as shown in Figure 3, was purified and directly sequenced using the ITS1 and ITS4 primers.

Table 2. Diameter of clear zones of protease produced from isolates

Isolates	Samples	Diameter of clear zones (mm)	Enzyme activity (U/L)
TB1	Soy sauce	17.0	49.26
TB2		11.0	29.10
NM2	Fish sauce	9.5	13.73
NM3		10.4	14.21
NM4		8.9	12.89
MS1	Miso sauce	8.5	16.90
MS3		8.2	16.20
MS4		7.6	15.74
DX3	Dau xi sauce	8.5	14.31
DX4		6.4	11.31
TC1	Fermented shrimp	6.5	13.49
TC3		5.1	7.24

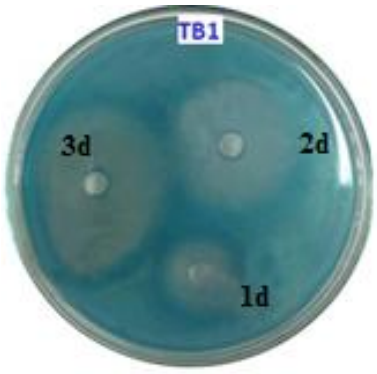


Figure 2. Casein degradation of TB1 protease on an agar plate flooded with BCG reagent after 3 days of incubation (1 d, 2d, 3d: after 1 day, 2 days, 3 days of enzyme incubation, respectively)

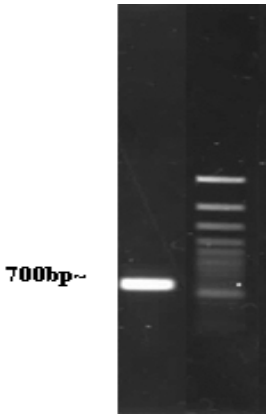


Figure 3. PCR product of the ITS region from strain TB1, showing a 700bp fragment. Lane M, DNA ladder (100bp DNA ladder, New England Biolabs)

Homologous sequences of the ITS sequence of the strain TB1 were searched for in the GenBank database using the BLAST program. The BLAST searches showed that the TB1 strain was most closely related to an *A. oryzae* originating from a wide range of sources throughout the world. Based on the analysis of the ITS sequence, TB1 was identified and named to be *Aspergillus oryzae* TB1 (*A. oryzae* TB1).

3.4. Determination of suitable cultivation time on protease production

In tests to find out the suitable cultivation time to harvest protease of *A. oryzae* TB1, protease activity increased gradually and reached a maximum value at 72 hours of incubation, and then decreased with time (Fig. 4). A 72-hour cultivation time was used to produce enzyme extract for further study.

Effect of pH on protease activity and stability: The optimum pH for protease activity in *A. oryzae* TB1 was determined over a pH range from 3.0 to 9.0. The results, as shown in Figure 4a, showed that protease from *A. oryzae* TB1 was more active at a neutral pH than in acidic or alkaline conditions, and the optimum pH was revealed to be pH 7.0. This result was comparable with that of Sandhya *et al.*, 2005 and Wang *et al.*, 2013 who showed that protease from *Apergillus* sp. exhibited maximum activity in the pH range 7.0 - 8.

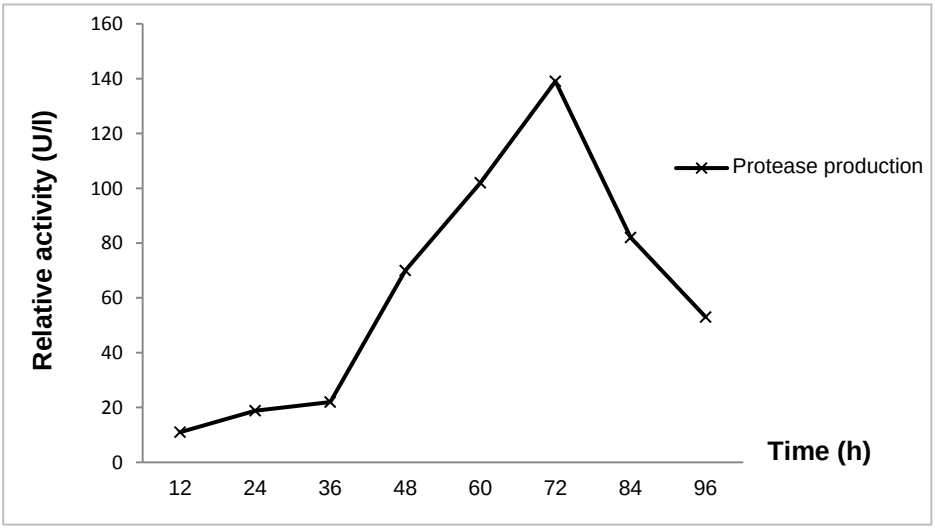
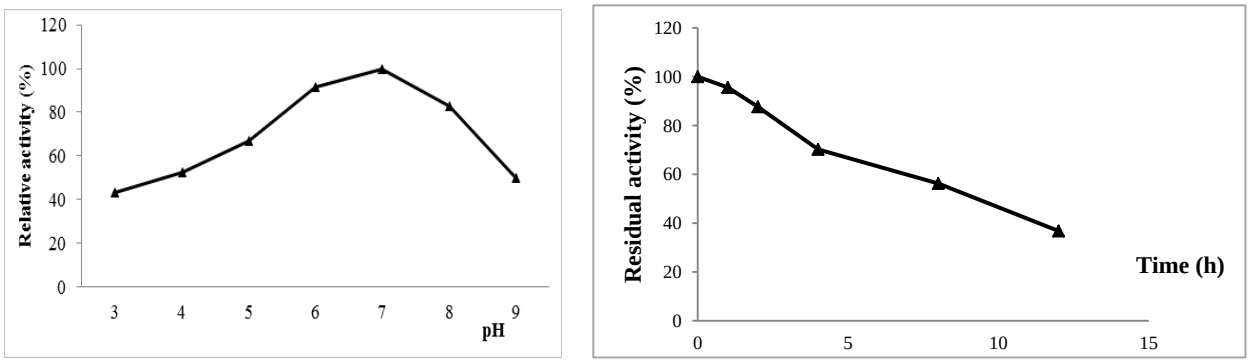


Figure 4. Effect of incubation time on protease production of *A. oryzae* TB1



(a) (b)

Figure 4. Effects of pH on the activity (a) and stability (b) of the protease produced from *A. oryzae* TB1

Protease stability was expressed as residual activity after incubation at pH 7.0 for 12h at 37°C with 100% referring to the initial activity. In general, protease activity from TB1 tended to decrease under the mentioned conditions (Figure 4b). Protease activity was maintained over 50% and 37% of its original activity after 8 hours and 12 hours of incubation, respectively.

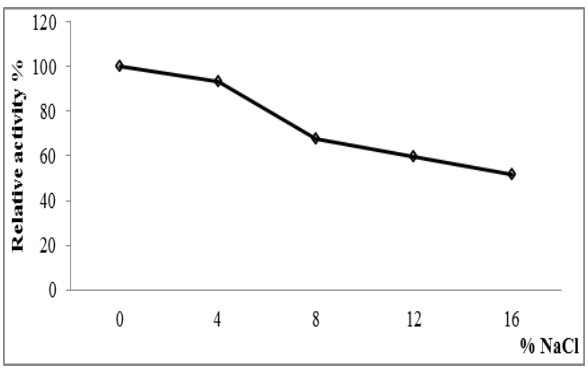
Effect of sodium chloride (NaCl) on protease activity and stability: In food fermentation, such as in koji or sauce production, a protease capable of high salt concentration tolerance is very necessary. In a recent study, Mueda (2015) revealed that the sodium chloride content found in commercial fish sauce was around 16 - 20 %.

The effect of sodium chloride on protease activity of crude enzyme extracts from *A. oryzae* TB1 is shown in Figure 5a. The concentration of sodium chloride significantly affected protease activity of *A. oryzae* TB1. The higher the concentration of sodium chloride, the lower the enzymatic acitvity. These results were quite compatible with a study of Wang *et al*, 2013 which revealed the residual activity of protease decreased sharply with the increase of NaCl concentrations from 0% to 16%.

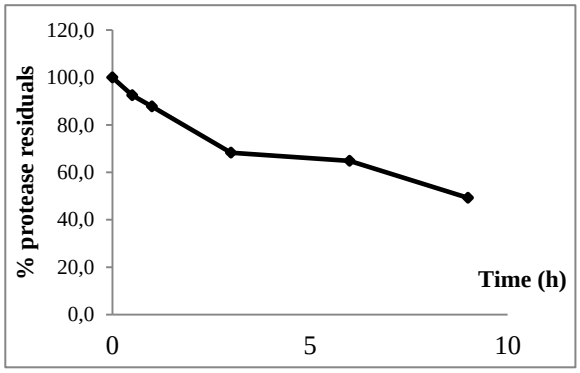
The salt tolerance of protease is a very important property in soy sauce production, and it is related to the length of fermentation time and the utilization of raw materials. The tolerance of protease activity from *A. oryzae* TB1 was determined at 16% NaCl (w/v) concentration, pH 7.0 and 30°C. As shown in Figure 5b, TB1 protease activity decreased slowly to 49.2% after 9 hours of incubation. The results showed that protease activity would decrease as the ionic strength increased. As a result, the protein had the tendency to change its conformation to reduce exposure of its surface by the salting out mechanism (Wang *et al.*, 2013). With 9 hours of salt tolerance, the protease in this study showed feasiblity to be used in short-length fermented products such as koji. Many researchers have studied the fractionation of proteases from koji molds and demonstrated their roles in soy sauce manufacturing. However, all of these protease activities declined significantly when the harvested koji was incubated with a high level of sodium chloride (~16-18% NaCl) to make a mash (Su *et al.*, 2005).

4. CONCLUSIONS

In this paper, we described the identification and characterization of a crude protease from *A. oryzae* TB1 isolated from soy sauce. Protease activity of this strain was highly active in the pH range of 5.0 - 8.0, especially at pH 7.0. Furthermore, after 9 hours of enzyme incubation at a 16% NaCl concentration (w/v), 49.2% of residual activity still remained. Consequently, this high salt tolerant protease is a new potential enzyme for soy sauce production and other relevant applications under high salt conditions.



(a)



(b)

Figure 5. Effects of NaCl on TB1 protease activity (a) and stability (at 16% NaCl) (b)

Acknowledgement

The authors are thankful to the program "Masters in Food Technology, Safety and Quality Management" funded by Académie de Recherche et d'Enseignement supérieur - Commission de la Coopération au Développement (ARES - CCD), for the support of research grants to complete this work.

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MODELING APPROACH FOR DETERMINING THE BIOLOGICAL AGE OF TOMATO FRUITS 'CV.SAVIOR' GROWN DURING THE WINTER

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Received date: 04.12.2016 Accepted date: 14.07.2017

ABSTRACT

Tomato is a worldwide economic valuable and healthy crop with good nutritional properties. However, postharvest losses of tomato are relatively huge due to suboptimal harvest techniques and the maturity index. This issue could be solved if the biological variation of a tomato cultivar is quantified. In this study, a mathematical model was established to determine the biological age of tomato fruit cv 'Savior' grown during the winter. After which, the model was successfully validated using a single point estimation method. The model parameters estimated in this study could be used to predict the biological age of tomato grown in different locations and periods. The data play an important role for predicting the optimal harvest strategy in further research.

Keywords: Biological age, Fruit development, *Lycopersicon esculentum,* Modeling, Tomato, Ripening

Thiết lập mô hình toán học để xác định tuổi sinh học của quả cà chua giống savior trồng vụ đông

TÓM TẮT

Cà chua là một loại rau ăn quả được sử dụng rất phổ biến để ăn tươi cũng như làm nguyên liệu cho công nghiệp chế biến. Tuy nhiên tổn thất sau thu hoạch của cà chua khá cao do kỹ thuật thu hái và độ chín của cà chua khi thu hái chưa được tối ưu hóa. Vấn đề này có thể giải quyết nếu thông tin về dao động sinh học của quả cà chua được định lượng. Trong nghiên cứu này mô hình toán học được thiết lập nhằm xác định tuổi sinh học cho quả cà chua giống Savior trồng vụ đông. Tiếp theo, mô hình đã được kiểm định bằng phương pháp ước lượng điểm đơn và cho kết quả tốt. Các thông số của mô hình ước lượng trong nghiên cứu này có thể được dùng để dự đoán tuổi sinh học của giống cà chua này khi trồng ở các địa điểm và thời điểm khác nhau. Dữ liệu này đóng vai trò quan trọng trong nghiên cứu tiếp theo, cho phép xác định thời điểm thu hái tối ưu cho giống cà chua Savior trồng vụ đông.

Từ khóa: Cà chua, *Lycopersicon esculentum*, mô hình hóa, sự phát triển và chín của quả, tuổi sinh học.

1. INTRODUCTION

Tomato, *Lycopersicon esculentum* Mill, is a worldwide economically valuable and healthy crop with good nutritional properties. In Vietnam, tomato was first introduced about 100 years ago. The production area has been increasingly expanding in recent decades as tomato has become an important export crop. As a result, a farmer's income from tomato cultivation is 4-times higher than that from rice cultivating. Vietnam's tomato production area is about 15,000 - 17,000 ha with the yield ranging from 45-60 tons/ha (Tran Duc Vien, 2006).

Currently, there are two main types of tomato cultivars being cultivated in Vietnam: traditional heat sensitive cultivars and new heat tolerant cultivars. The latter are widely grown in the North of Vietnam as they are able to set fruit in high temperatures so the farmer can grow them both in winter and summer seasons. Among the heat tolerant cultivars, Savior is one of the most favored cultivars for

its high yield performance, good appearance, and popularity among consumers.

Although tomato production is important in Vietnam and quality cultivars have been introduced, postharvest losses of tomato are still huge as farmers are unable to define the optimal picking time that ensures a good postharvest life of fruits. They mostly decide the picking time based on the date after anthesis and fruit color. However, fruit apperance only relatively describes the physiological maturity. Moreover, the color based classification of tomato ripeness used currently is discrete and subjective, and does not takes into account the biological variation of individual fruits in a batch. As a consequence, some fruits in a batch are harvested so early that they often fail to ripen while others are harvested so late they are unable to withstand being handled in the supply chain. Hence, there is an urgent need to find a method for predicting the optimal harvest time that is science-based and objective to meet the stringent retail demands for continuity of high quality products. A good approach for determining the biological age is crutial to build up a optimal harvest model for tomato fruit.

There have been some research groups using the biological age to classify the maturity of different fruits such as tomato (Hertog *et al.*, 2004), nectarines (Tijskens *et al.*, 2007; Rizzolo *et al.*, 2009), apple (Tijskens *et al.*, 2008, 2009). Recently, Van de Poel *et al.* (2012) expanded the biological age concept and used it to study other quality atributes of tomato (cv. Bonaparte) during development and ripening. In this study, we aimed to apply a similar approach to a larger population to determine the biological age of tomato fruit (cv. Savior).

2. MATERIALS AND METHODS

2.1. Plant material

Tomato seedlings (cv. Savior) were transplanted in the open field during the 2014 winter season at the Fruit and Vegetables Research Institute, Hanoi, Vietnam (21°00'38.9"N 105°55'39.2"E). From 300 randomly chosen plants, 700 tomato flowers were labeled on the plants shortly after anthesis across three labeling periods with 5-day intervals, each to cover wide range of fruit variation. Then, 360 individual fruits were selected to be monitored for color and diameter on the plants at three-day intervals during fruit development and two-day intervals during fruit ripening.

2.2. Experimental measurements

2.2.1. Fruit mass

Fruit diameter was monitored on-plant using a caliper (Mitutoyo, Japan). Fruit mass was calculated from fruit diameter and an average fruit density of 0.873 g/cm^3 as below:

$$m = V \times d = \frac{4}{3}\pi \times \left(\frac{D}{2}\right)^3 \times 0.873$$

where m: the fruit mass (g); V: the fruit volume (cm^3); D: the fruit diameter (cm); and d: the fruit density (g/cm^3)

2.2.2. Fruit skin color

The fruit skin color was measured on the same spot, at the equator of each fruit, using a Minolta CM-2500d colorimeter (Minolta Camera Co., Ltd, Osaka, Japan), and expressed in the CIELAB color space L*, a*, and b*. The fruit color was characterized in hue angle (°).

$$H = \arctan\left(\frac{b^*}{a^*}\right)$$

2.3. Model development

2.3.1. Fruit growth model

The change of fruit mass (*M (g)*) over time was modeled using the standard Gompertz growth model (Winsor, 1932) in its differential form (Eq. (1)):

$$\begin{cases} M(t) = M_{\max}.\exp(-C.\exp(-k_m.t)) \\ \dfrac{d}{dt}M(t) = k_m.M.\ln\left(\dfrac{M_{\max}}{M}\right) \\ M(0) = M_{\max}.\exp(-C) \end{cases} \quad (1)$$

where k_m (d^{-1}): the growth rate; M_{max} (g): the maximum fruit mass; and C: a dimensionless displacement factor from the Gompertz

function. These parameters were estimated from Eq. (1).

It was assumed that k_m and C were the variables whose values were generic for a specific cultivar while M_{max} was assumed to be different for every fruit. The Gompertz model was proven most suited given preliminary trials with several other growth models (Van de Poel *et al.*, 2012).

2.3.2. Color change model

Once a fruit has almost reached its maximum size, color change is triggered. Hue color change (measured as H in◦) was described using a simple exponential decay model (Eq. (2)) which was implemented in its differential form:

$$\begin{cases} H(t) = H_{\min} + (H_O - H_{\min}).\exp(-k_h \times t) \\ \dfrac{d}{dt}H(t) = -(H - H\ \min).k_h \\ H(0) = H_o \end{cases} \quad (2)$$

where k_h (d^{-1}): the rate of color change; H_{min} (º): the minimum hue value; and H_o (º): the initial hue value. The parameters k_h, H_{min}, and H_o were assumed to be generic for a specific cultivar and were estimated from Eq. (2).

2.3.3. Biological switch model

The experimental data revealed that color change is only initiated after the fruit approachs its maximum size. By using the mass and color change data, the relationship between biological switch and the rate constant k_h is described by Eq. (3):

$$k_h = \frac{k_h^{\max}}{(1 + ((M_{\max} - M)/M_{\max}))^s} \quad (3)$$

where k_h^{max} (d^{-1}): the maximum rate of color change once fully triggered; and s: (dimensionless) defines the steepness of the switch and k_h^{max}. These two parameters were estimated using Eq. (3).

2.3.4. Biological age

The combination of fruit mass and fruit skin color is a good indicator of the biological age of an individual fruit. While the time of harvest only provides an arbitrary starting time point, biological age puts the experimentally measured values along a standardized time scale relative to a common development pattern. Biological age (t_{age} in d) is calculated from the experimental time values (t_{exp} in d, relative to the day of harvest) by adding a constant correction factor (Δt in d) following Eq. (4).

$$t_{age} = t_{\exp} + \Delta t \quad (4)$$

The correction factor Δt is a fruit specific factor. At harvest, $t_{exp} = 0$, $t_{age} = \Delta t$.

2.4. Model calibration using time series based data

The integrated models (Eqs. (1)-(4); with t being t_{age}) were calibrated using the dataset of color and mass collected during fruit development and ripening. The model parameters were estimated using OptiPa (Hertog *et al.*, 2007), a dedicated freeware optimization tool which was developed for use with Matlab (Matlab R2007b, The Math-Works, Inc., Natick, MA, USA). Based on the fruit mass data, common values for k_m, C; and fruit specific values for M_{max} và Δt were estimated. Then, based on the fruit skin color data, common values for k_h^{max}, H_{min}, H_o were estimated.

2.5. Model validation using single point estimation based data

Model validation using single point estimation was performed according to the procedure described by Van de Poel *et al.* (2012). The entire time series dataset, which was used to calibrate the model in the previous step, was artificially fractionated. The time series were broken apart and considered as 7857 individual points. Afterward, Δt values were estimated by the same analysis approach on the 7857 single observations. Finally, the resulting t_{age} values were compared to their t_{age} values obtained when calibrating the model.

3. RESULTS AND DISCUSSION

3.1. Change of mass during fruit development and ripening

The change of mass during fruit development and ripening is indicated in Figs. 1 - 3. All fruit followed an identical growth pattern. The initial growth process was slow and characterized mainly by cell division. Subsequently, cell expansion took place by which fruitlets grew faster mainly due to water uptake until some variable of maximal mass was reached. The development stage for Savior took about 50 - 52 d after anthesis. It was observed that different fruit reached a wide range of masses (from 30 g to 180 g) despite their similar flowering time. This can be explained by the fact that fruit are exposed to different microclimate conditions and sink/source relations within the plant (Van de Poel *et al.*, 2012). Therefore, M_{max} was estimated for every single fruit when calibrating the model.

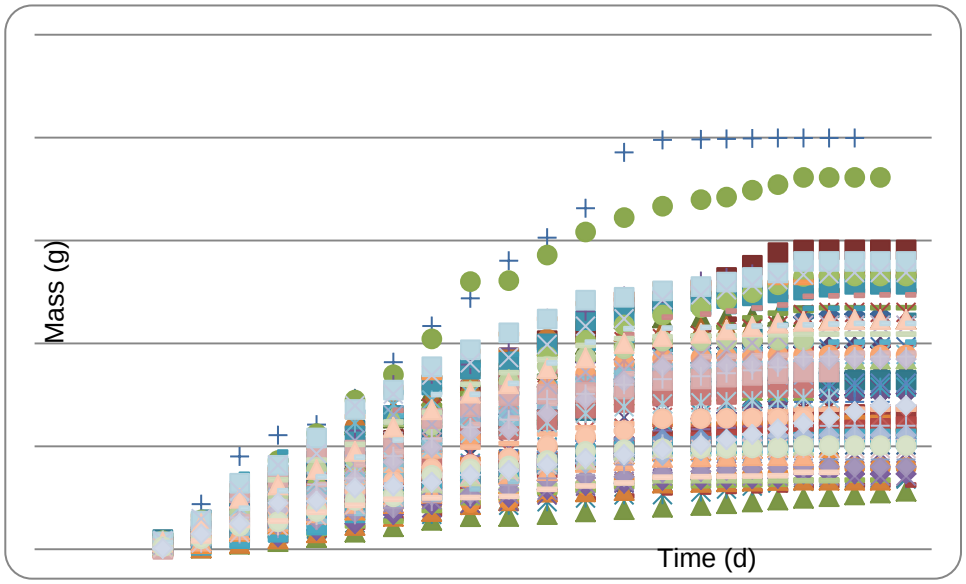

Fig. 1. Change of mass during fruit development and ripening (1st labeling period)

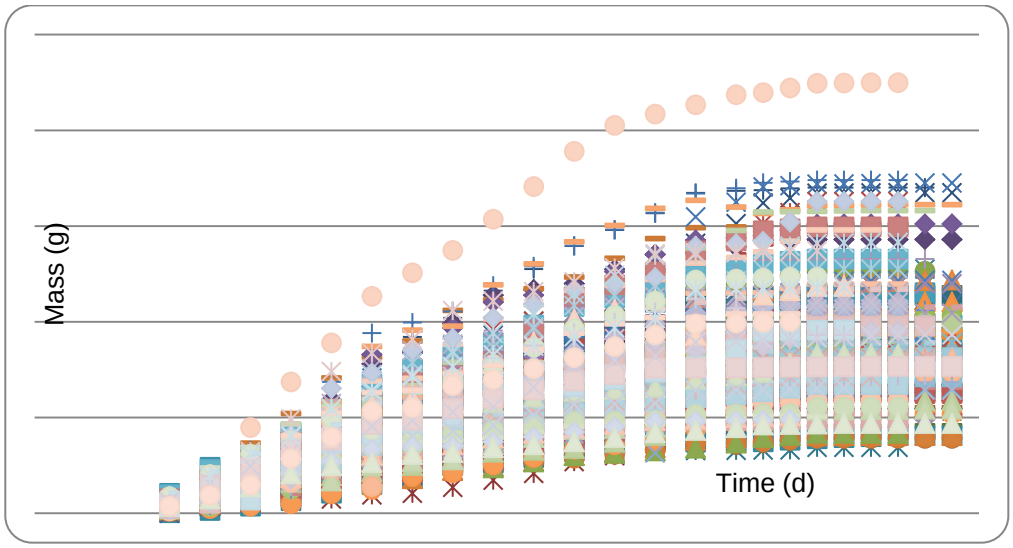

Fig. 2. Change of mass during fruit development and ripening (2nd labeling period)

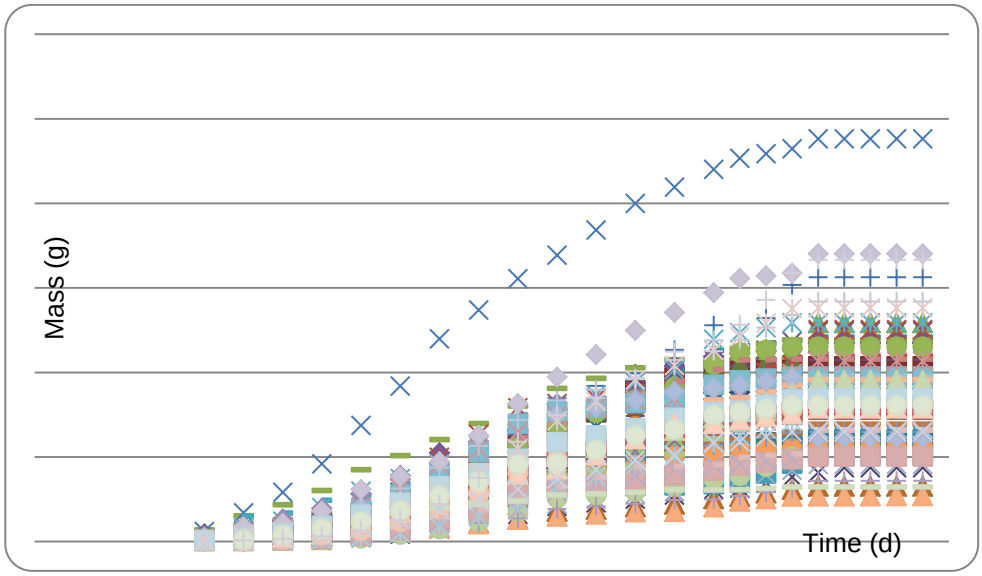

Fig. 3. Change of mass during fruit development and ripening (3rd labeling period)

3.2. Change of skin color during fruit development and ripening

The change of fruit skin color is illustrated in Figs. 4 - 6. During fruit development, there was no change of the hue value with the green fruit color being determined by chlorophyll. Color change was only triggered after the fruit approached its final mass. While mass remained almost constant, color dropped from immature green (hue ranging from 115° to 119°) down to mature red (hue ranging from 42° to 55°). Moreover, the color data revealed that there was a shift along the time axis among fruit, indicating that the biological age of individual fruits at the transition stage are not completely identical. Therefore, fruit specific values for Δt were estimated as well.

3.3. Model calibration using time series data

By using both mass and color data from the time series based dataset, the integrated model was calibrated by estimating the various model parameters. The goodness of fit is illustrated in Fig. 7. The generic parameters for the specific cultivar are given in Table 1, while the fruit specific parameters are given in Fig. 8.

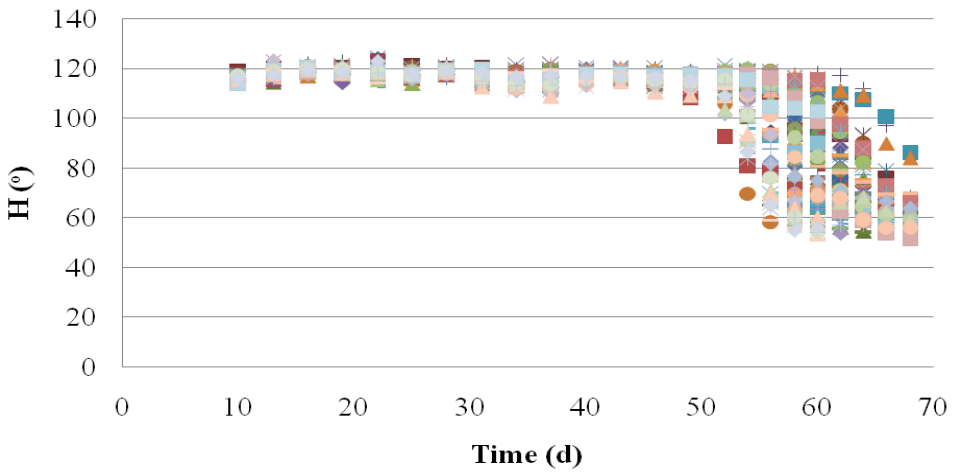

Fig. 4. Change of fruit color during fruit development and ripening (1st labeling period)

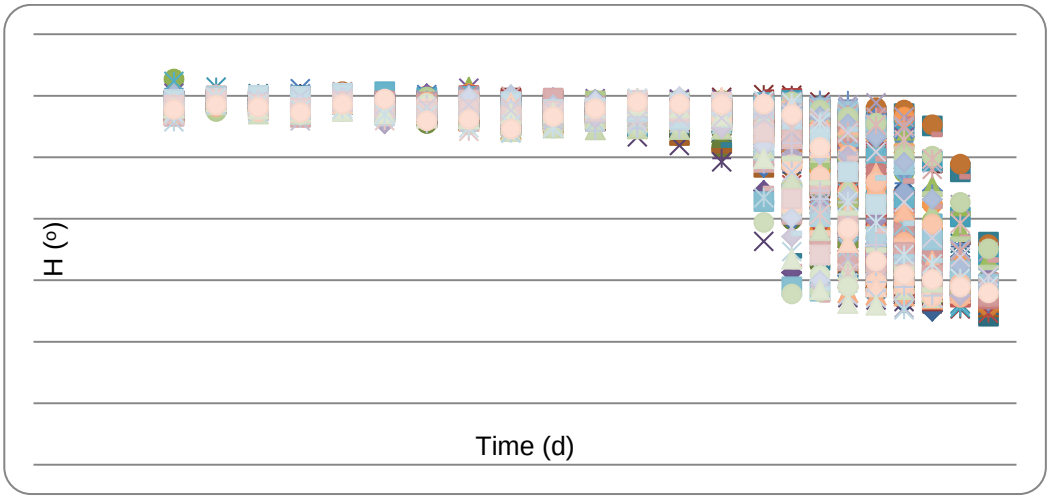

Fig. 5. Change of fruit color during fruit development and ripening (2nd labeling period)

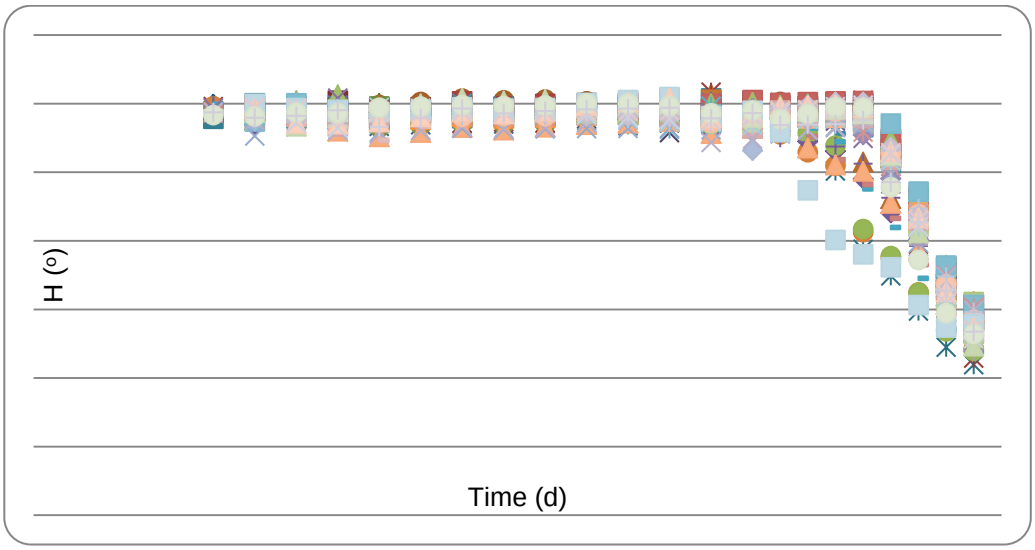

Fig. 6. Change of fruit color during fruit development and ripening (3rd labeling period)

Table 1. Parameter estimates for the calibration of the integrated model (Eqs. (1)-(4) fitted to the dataset of mass and color (n = 360)

Parameter	Estimate	Standard deviation
Growth model parameters		
C	6.54	0.05E-4
k_m (d^{-1})	0.0724	0.00004
Color change model parameters		
k_h^{max} (d^{-1})	80.38	4.79
H_o (°)	116.81	0.04
H_{min} (°)	58.19	0.16
Biological switch parameter		
s	73.22	0.67

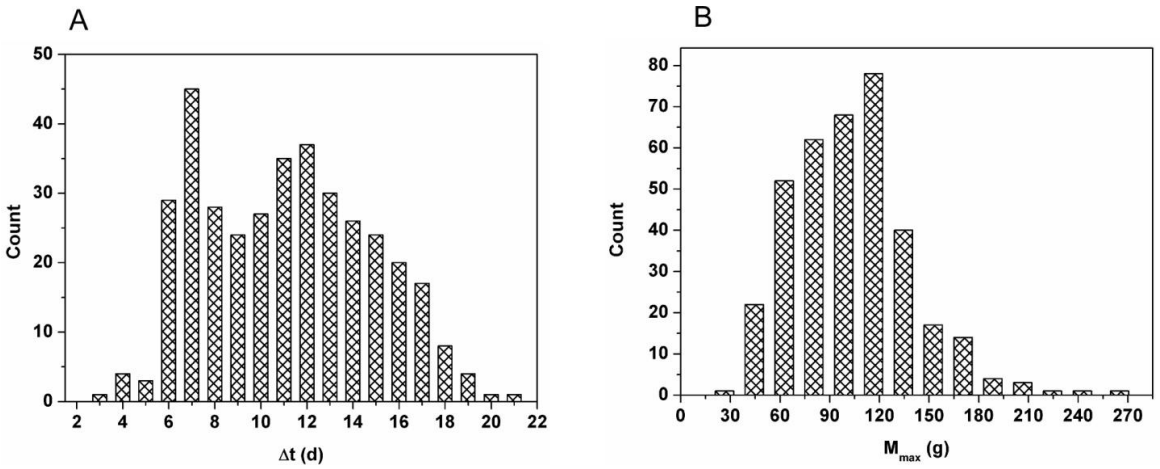

Fig. 7. Histograms showing A) the distributions of estimated Δt (d) for the 360 fruit from the dataset, and B) the distributions of estimated M_{max} (g) for the 360 fruit from the dataset

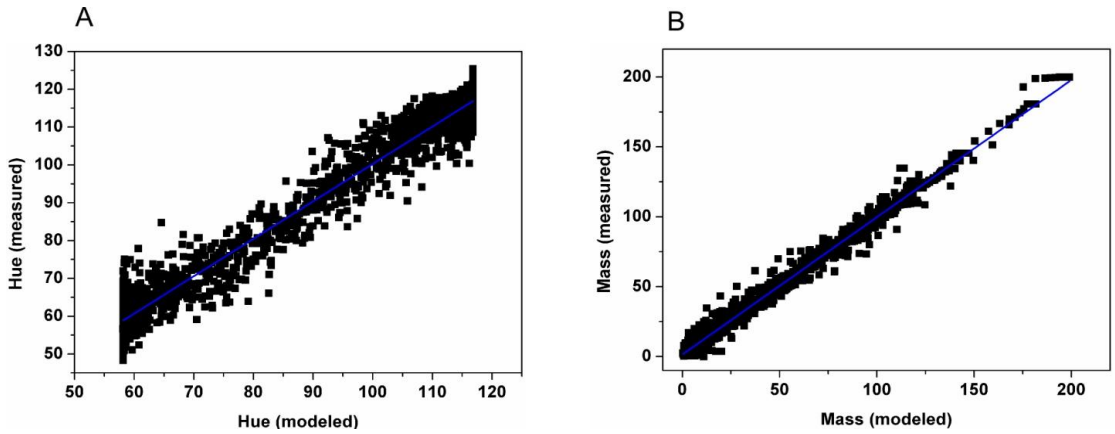

Fig. 8. A) Measured versus modeled color values, and B) Measured versus modeled mass values showing the goodness of fit for the integrated model, calibrated on the mass and color measurements of the dataset. The straight lines represent the line of perfect agreement between the modeled and measured values

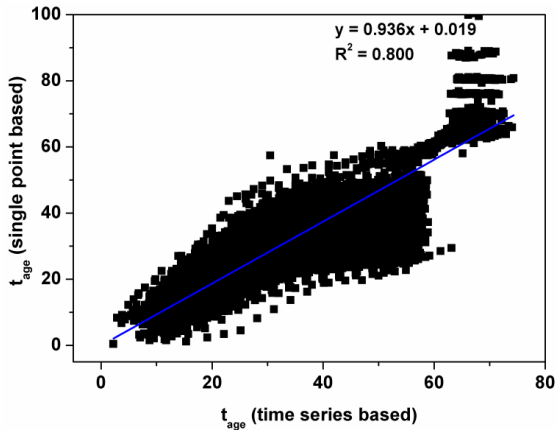

Fig. 9. Scatter plot showing the t_{age} (d) obtained by the time series based versus t_{age} (d) obtained by single point based

Fig. 7 shows that the overall fit of the model was good, the coefficients of determinations were 0.975 and 0.986 for color and mass, respectively, explaining about 98% of the observed variation in both fruit mass and hue color data. Data in Table 1 show that the generic parameters were estimated very accurately demonstrated by their small standard deviation. This was probably due to the large number of observations used (n = 360). When a comparison was made between the generic data obtained for Savior in this study and Bonaparte grown in a glass house in Belgium by Van de Poel *et al.* (2012), the standard deviation of some parameters such as C, k_h^{max}, and s for Bonaparte were higher than Savior as they had lower a number of observations (n = 60). Moreover, while both cultivars had similar magnitudes for most generic parameter estimates; Bonaparte had a higher value for s and lower values for k_h^{max} than Savior. The higher value of s means that the color change of Bonaparte is only triggered towards the end of the growth cycle. For k_h^{max}, a higher value was obtained for Savior (80.38 d^{-1}) than for Bonaparte (57.7 d^{-1}). This is because Savior was grown in an open field that gets more sunlight than Bonaparte grown in a glass house; hence, the rate of color change was higher. A similar phenomenon was observed for Savior grown at the same period in the net house (54.95 d^{-1}).

The distribution of the fruit specific parameters M_{max} and Δt are shown in Fig. 8. The maximum fruit mass M_{max} ranged from 26.5 g to 265 g with a mean of 93.91 ± 35.83 (g), confirming the broad range of fruit masses encountered (Figs. 1 - 3). The Δt ranged from 3 to 21 days with a mean of 10.60 ± 3.65 9 (d) (Fig. 8). From these findings, it can be concluded that the current approach can be applied for different tomato cultivars but it is necessary to estimate the generic parameters for each specific cultivar.

3.4. Model validation using single point estimate

Model validation using single point estimation was performed by fractionating the time series data into 7857 individual points; each was a combination of mass and hue value. Subsequently, using the same integrated models (Eqs. (1)-(4)) and fixed generic parameters, only the t_{age} values were estimated for 7857 observations. These t_{age} data were then compared with those obtained after rescaling the time series data using Eq. (4) and Δt for each fruit. The results are given in Fig. 9.

As the slope of the fitted line is 0.936, a high correlation between t_{age} was obtained between the time series based data and the single point based data. In addition, the coefficient of determinations was 0.8 for t_{age}, indicating that the fit was quite good. Eighty percent of the total variation in one variable can be explained by variation in the other variable. However, the fit still shows some deviations from the ideal 45◦ line, especially for bigger green fruits for which a good estimate of M_{max} is essential in order to obtain a good estimate of t_{age}, and for the fully red fruit, at which time one can no longer discriminate between fruits based on mass and/or color. The range in which the model works well is when fruit are either small or have undergone a color change, which is exactly the time all the postharvest sciences are interested in. In conclusion, the validity of the model was confirmed.

4. CONCLUSIONS

In this study, the changes in mass and skin color of 370 tomato fruits cv. 'Savior' grown during the winter season were monitored during fruit development and ripening. There were variations in color and especially in the masses of fruits having the same flowering time. The full measurement dataset was used to calibrate the model. Next, the model was successfully validated using a single point estimation method.

In further research, the calibrated model developed in the current study will be combined with data on quality changes of tomatoes during fruit development and ripening in order to build up a population model for predicting the optimal harvest date.

Acknowledgements

This research was performed in the framework of the Bilateral Scientific Research Cooperation Projects FWO.106.2013.20 between NAFOSTED (Vietnam) and FWO (Flander, Belgium). NAFOSTED is gratefully acknowledged for their financial support. The authors thank the staff and students of the Faculty of Food Science and Technology, Vietnam National University of Agriculture, and Fruits and Vegetables Research Institute, Vietnam for their kind assistance.

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EXPRESSION OF CHITINASE GENE FROM *Bacillus Licheniformis* DSM13 IN *E.Coli* T7 AND BIOCHEMICAL CHARACTERIZATION OF RECOMBINANT ENZYME

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Received date: 26.07.2017 Accepted date: 05.09.2017

ABSTRACT

In this study, the mature gene encoding for the enzyme endochitinase from the Gram positive bacterium *Bacillus licheniformis* DSM13 (ATCC 14580) was cloned into the expression vector pET-21d, and then overexpressed in *E. coli* T7. A high expression level was indicated by SDS-PAGE and an enzyme activity assay using pNP-$(GlcNAc)_2$ 0.18 mM as the substrate. The activity of the enzyme expressed in *E. coli* T7 was approximately 8 times higher than that previously expressed in *Lactobacillus plantarum*. Recombinant chitinase was purified from cell extract and characterized using coloidal chitin 2% as the substrate. The enzyme showed good thermostability (half-lives of 15 days and 8 days at 37 and 50^o C, respectively), and good stability in the pH range of 5 - 9. The main product of colloidal chitin hydrolysis as indicated by thin layer chromatography was diacetyl glucosamine. The results demonstrated that this enzyme was promising for chitin waste bioconversion into different chitin-oligosaccharides, such as functional diacetyl glucosamine.

Keywords: *Bacillus licheniformis* DSM13, chitinase, *E. coli* T7.

Biểu hiện gene mã hóa chitinase từ *Bacillus licheniformis* DSM13 trong *E.coli* T7 và xác định đặc tính của enzyme tái tổ hợp

TÓM TẮT

Trong nghiên cứu này, gene mã hóa cho enzyme endochitinase của vi khuẩn gram dương *Bacillus licheniformis* DSM13 (ATCC 14580) được chuyển vào vectơ biểu hiện và biểu hiện trong tế bào *E. coli* T7. Mức độ biểu hiện cao của enzyme được thể hiện qua SDS-PAGE và hoạt tính của enzyme khi sử dụng pNP-$(GlcNAc)_2$ 0,18 mM là cơ chất. Hoạt tính của enzyme được biểu hiện trong *E.coli* T7 là cao khoảng gấp 8 lần so với enzyme biểu hiện ở *Lactobacillus plantarum* (nghiên cứu trước). Chitinase tái tổ hợp được tinh sạch và xác định đặc tính bằng cách sử dụng chitin 2% là cơ chất. Kết quả chỉ ra rằng, enzyme tái tổ hợp bền nhiệt (enzyme còn lại một nửa hoạt tính sau 15 và 8 ngày ủ ở 37 và 50^{o}C tương ứng), bền ở giải pH từ 5 - 9. Sản phẩm chính thủy phân chitin huyền phù được xác định bằng phương pháp sắc ký bản mỏng là diacetyl glucucosamine. Các kết quả nghiên cứu này chỉ ra rằng enzyme tái tổ hợp này có tiềm năng trong việc ứng dụng sản xuất chitin-oligosaccharide, như là diacetyl glucucosamine chức năng từ việc chuyển hóa phế phụ phẩm chitin

Từ khóa: *Bacillus licheniformis* DSM13, chitinase, *E.coli* T7.

1. INTRODUCTION

Chitinases (EC 3.2.1.14) are glycosyl hydrolases that catalyze the hydrolytic degradation of chitin, which is an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine (GlcNAc), and is the second most abundant polysaccharide in nature after cellulose (Kurita, 2001). Chitin is widely distributed in nature and forms a major constituent of the shells of crustaceans such as crabs, shrimps, lobster, and squid, which are

readily available in huge amounts in countries with a strong aquaculture industry such as Vietnam (Nguyen *et al.*, 2011). This waste material causes environmental pollution if not processed and disposed of properly, but could serve as a valuable chitin source for the production of chitin-oligosaccharide functional food ingredients.

Chitinases have been purified from many sources and their enzymatic activities have been investigated. This includes plants, fungi, yeasts, bacteria, insects, and even vertebrates (Clarke and Tracey, 1956; Hsu and Lockwood, 1975; Htakara *et al.*, 1979; Kramer and Koga, 1986; Hearn *et al.*, 1996; Kasprzewska, 2003; Ajit *et al.*, 2006; Akagi *et al.*, 2006). Chitinase from *Bacillus* has received considerable attention as it often has high activity and thermostable characteristics, especially *Bacillus licheniformis* (Barboza-Corona *et al.*, 1999; Toharisman *et al.*, 2005; Yamabhai *et al.*, 2008; Songsiriritthigul *et al.*, 2010;). The complete genome of *Bacillus licheniformis* DSM 13 has been elucidated and was found to contain a number of genes coding for polysaccharide-degrading enzymes including a gene for chitinase (glycoside hydrolase family GH18) with the GenBank accession number AAU21943 (Veith *et al.*, 2004). This gene has been cloned and expressed in *Lactobacillus plantarum* using the pSIP vector, and the recombinant chitinase was purified and characterized. Results indicated that this chitinase was a promising enzyme for the production of chitin-oligosaccharides (Nguyen *et al.*, 2011). However, the main drawback was the fact that the expression level of the enzyme was quite low (*ca.* 5 mg of recombinant protein per liter of cultivation medium), and thus, it is impossible to apply in industrial scale.

The aim of this study was to enhance the expression of the chitinase gene (AAU21943) from *B. licheniformis* DSM13 by using the pET-21d expression vector in *E. coli* T7. The recombinant enzyme was also purified and characterized in order to find the effect of the new expression system on enzyme characteristics.

2. MATERIALS AND METHODS

2.1. Enzymes, substrates and chemicals

Restriction enzymes and T4 DNA ligase were supplied by Fermentas (St. Leon-Rot, Germany). Isopropyl-β-D-thiogalactoside (IPTG) was from Roth (Karlsruhe, Germany). Chromogenic substrates, *p*NP-$(GlcNAc)_2$, and chitin were purchased from Sigma Aldrich (St. Louis, MO). Diacetyl chitobiose was purchased from Megazyme (Bray, Ireland). All other chemicals were of reagent grade and obtained from commercial sources.

2.2. Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Roberts and Selitrennikoff (1988) with slight modifications. Briefly, 5 g of chitin from crab shells (C7170, Sigma Aldrich) was gradually added into 100 ml of cold concentrated HCl with gentle agitation on a magnetic stirrer for 18 h at 4°C. The mixture was then added to 500 ml of ice-cold 96% ethanol and left for 24 h with rapid stirring at 4°C. The precipitate was harvested by centrifugation at 8000 g for 20 min at 4°C and washed repeatedly with sterile distilled water until the pH reached 6. The colloidal chitin was kept at 4°C until further use. Approximately 95 ± 4 g of colloidal chitin was obtained by this procedure from 5 g of chitin powder.

2.3. Bacterial strains, plasmids, and media

Bacillus licheniformis DSM13 (=ATCC 14580, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was grown in M1 medium at 37°C with shaking at 125 rpm. Genomic DNA was extracted using the DNA Isolation Kit (Norgen, Thorold, Canada). Plasmid pET-21d (Novagen, Merck, Darmstadt, Germany) was used as the expression vector. *E. coli* T7 One Shot Chemically Competent (Invitrogen, Carlsbad, CA) was used as the expression host. *E. coli* T7 carrying pET-21d with the gene of interest was grown in LB broth with ampicillin (100 µg/ml) for expression of the recombinant protein.

2.4. Cloning and expression of the chitinase encoding gene

A forward primer (GCGGCCATGGATTCCGGAAAAAACTAT) and a reverse primer (TAATCTCGAGTTCGCAGCCTCCGATCAGCC) containing *Nco*I and *Xho*I recognition sites (underlined), respectively, were designed based on the sequence of a gene encoding a chitinase from *Bacillus licheniformis* with the GenBank accession No. AAU21943 (Veith *et al.*, 2004). Amplification conditions for a 25 µl standard PCR reaction were as follows: 1 cycle at 98°C for 3 min; 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min; and an extra extension at 72°C for 5 min for the final cycle. The amplification product was purified from an agarose gel using the Wizard SV Gel PCR Cleanup system (Promega, Madison, WI) and digested with *Nco*I and *Xho*I before directly being cloned into the *Nco*I and *Xho*I sites of pET-21d. Insertion of the amplicon resulted in translational fusion of the chitinase open reading frame with the vector-encoded 6x His-tag (N-terminal tag). Plasmids were transformed into *E. coli* T7. Positive colonies were confirmed by colony PCR and DNA sequencing. After culturing, cells were stored in 30% glycerol at -80°C.

Overnight cultures of *E. coli* T7 harboring pET-21d with the chitinase gene were used to inoculate 250 mL of fresh LB medium supplemented with 100 µg/mL of ampicillin. The cultures were incubated at 37°C and 200 rpm until the OD_{600} reached 0.5. IPTG was added to the cultures to a final concentration of 0.4 mM. Post-induction cultivation was done at 18°C and 25°C, 200 rpm for 16 hours. The cells were harvested by centrifugation (6000 rpm, 20 min, 4°C) and washed two times with 50 mM sodium phosphate buffer pH 6. The biomass was stored at -20°C for protein purification

2.5. Protein purification

The cells were disrupted 3 times in a French press (Amicon, Jessup, MD) in 50 mM sodium phosphate buffer, pH 6. A cell free lysate was obtained by ultracentrifugation at 30,000 rpm for 30 min at 4°C. Protein purification was done by immobilized metal affinity chromatography (IMAC), as follows: 10 mL of crude protein extract was loaded onto a 15-mL column of Profinity IMAC Ni-Charged Resin (BioRad, Hercules, CA) pre-equilibrated with buffer A (Na_2HPO_4 20 mM, NaCl 0.5 M, imidazol 20 mM, pH 6.5). After washing the column with two column volumes of buffer A, the enzyme was eluted with a flow rate of 0.5 mL/min using a gradient from 0% to 100% of buffer B (Na_2HPO_4 20 mM, NaCl 0.5 M, imidazol 0.5 M, pH 6.5). Fractions containing the highest enzyme activity, detected using pNP-$(GlcNAc)_2$ as the substrate, were pooled, and then the buffer was exchanged to 50 mM sodium phosphate buffer pH 6.0. The samples were concentrated by 10 kDa cutoff Amicon Ultra Centrifugal filter tubes (Millipore, Billerica, MA). The purified enzyme was stored in 50 mM sodium phosphate buffer pH 6.0 at 4°C and used for characterization

2.6. Protein electrophoresis and molecular weight determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions (Laemmli *et al.*, 1970) was performed to verify the purity of the enzyme preparations and to determine the molecular mass of the enzyme using 10% gels and a PerfectBlue™ vertical electrophoresis system (Peqlab, Erlangen, Germany).

2.7. Enzyme assays and protein determination

Standard enzyme assay using pNP-$(GlcNAc)_2$ as substrate

Enzyme activity was determined using p-NP-chitobiose (pNP-$(GlcNAc)_2$) as substrate. The assay was performed in principle as described by Yamabhai *et al.* (2008) with some modifications. The reaction was initiated by adding 20 µl of enzyme solution to 100 µl of 0.18 mM pNP-$(Glc\text{-}NAc)_2$ in 50 mM sodium phosphate buffer pH 6, and then the mixture

was incubated for 30 min at 37°C and 600 rpm using an Eppendorf thermomixer. After incubation, the reaction was stopped by adding 480 µl of 0.5 M Na2CO3. p-Nitrophenol liberated during the reaction was determined spectrophotometrically at 405 nm. One unit of enzyme is defined as the amount of the enzyme releasing 1 µmol of p-nitrophenol per minute under the given conditions.

Enzyme assay using colloidal chitin as substrate

The reaction mixture consisted of 250 µl of a 2%-solution of colloidal chitin in 50 mM sodium phosphate buffer pH 6 and 250 µl of appropriately diluted enzyme solution. After incubation for 30 min at 37°C and 600 rpm on a Thermomixer Compact (Eppendorf; Hamburg, Germany), the reaction was stopped by heating it at 100°C for 10 min and then centrifuged at 10,000 rpm for 5 min. The concentration of reducing sugars in the supernatant was determined based on the dinitrosalicylic acid (DNS) method using Glc-NAc as the standard as described previously (Miler *et al.*, 1959). One unit of chitinase activity was defined as the amount of enzyme releasing one 1 µmol of reducing sugar per minute under the specified assay conditions. This assay was used as the standard enzyme assay for the characterization of recombinant chitinase.

2.7. Protein determination

Protein concentration was determined according to Bradford (1976) using the Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA), with bovine serum albumin as the standard.

2.8. Effect of temperature and pH on enzyme activity

The temperature optimum of the recombinant enzyme was determined using standard assay conditions with 2% colloidal chitin as the substrate in the temperature range from 20°C to 90°C. The thermal stability of the enzyme was studied by incubating the purified enzyme in 50 mM sodium phosphate buffer pH 6.0 at 37°C and 50°C. At certain time intervals, samples were taken and the residual activity was measured with colloidal chitin as the substrate under standard assay conditions.

The pH optimum was determined by the standard assay with 2% colloidal chitin in the pH range from 4 to 10 using Britton-Robinson buffers (20 mM sodium citrate, 20 mM sodium phosphate, and 20 mM borate, adjusted to the required pH with NaOH).

To determine the stability of chitinase at different pH, the purified enzyme was incubated at 37°C in Britton-Robinson buffers with various pH values. The remaining enzyme activity was measured at different time intervals using 2% colloidal chitin as the substrate under standard assay conditions.

2.9. Analysis of hydrolysis products

Chitinase-catalyzed hydrolysis of di-N-acetyl chitobiose $(GlcNAc)_2$ was followed by incubating 100 µl of a 10 mM-solution of the respective substrate in 50 mM sodium phosphate buffer pH 6 with 4 mU of purified enzyme, using an Eppendorf thermomixer set at 37°C and 600 rpm. Samples (10 µl) were taken at various time points and chitinase was inactivated by incubating samples at 100°C for 5 min.

Hydrolysis of colloidal chitin was studied using the reaction mixtures (960 µl) containing 2 or 20% of colloidal chitin together with 80 mU of purified chitinase. These mixtures were incubated at 37°C, 600 rpm using an Eppendorf thermomixer. Samples (20 µl) were taken regularly and the enzyme was inactivated as described above.

The products released by the chitinase from these carbohydrate substrates were analyzed using thin layer chromatography (TLC) based on methods of Rauvolfová *et al.* (2004). Aliquots (1 µl) of the reaction mixtures were loaded onto high-performance TLC silica plates (Kieselgel 60 F245, Merck) and run against a mobile phase of isopropanol/water/28% ammonia (7:2:1, v:v:v). Plates were dried and sprayed with 5% H_2SO_4 in ethanol, followed by baking at 220°C in an oven for 10 min to develop the spots on the TLC plates.

3. RESULTS

3.1. Overexpression of a chitinase-encoding gene from *Bacillus licheniformis* DSM13 in *E. coli* T7

The chitinase gene of *Bacillus licheniformis* DSM13 was successfully cloned into pET-21d and the protein was expressed in *E. coli* T7 driven by the T7 RNA polymerase promoter. Cells from a 250-mL culture induced with 0.4 mM IPTG at both 18°C and 25°C for 16 hours were disrupted and checked for gene expression level by using SDS-PAGE (Figure 1), enzyme activity using pNP-$(GlcNAc)_2$ 0.18 mM as a substrate, and specific activity (Table 1).

The presence of a large protein band with a molecular weight of approximately 65 kDa (Figure 1) indicated that the gene encoding for chitinase from *Bacillus licheniformis* DSM13 was successfully overexpressed in *E. coli* T7 using pET-21d. Volumetric activity and specific activity of the enzyme expressed at 18°C were 12.90 U/L and 0.97 U/mg, respectively, and the numbers were approximately two times higher than those obtained at 25°C (Table 1). These enzyme activity data agree well with the protein band intensities obtained by SDS-PAGE (Figure 1). These results also indicated that protein inclusion bodies might have formed during protein expressing at 25°°. The results presented in Figure 1 and Table 1 also demonstrated that chitinase expressed in *E. coli* at 18°C (12.9 U/l) was approximately 8 times higher than that reported previously by Nguyen *et al.* (2011) in *L. plantarum* (1.56 U/l). Chitinase obtained at 18°C was used for further purification and characterization.

3.2. Purification of recombinant chitinase

Cells from a 250-mL culture induced with 0.4 mM IPTG and grown for 16 hours at 18°C after induction were disrupted, and chitinase was purified from the cell free extract by one-step IMAC. The procedure yielded 68.2% chitinase recovery (Table 2). Typically, close to 20 mg of pure recombinant enzyme was obtained from 1 L of culture with a total activity of around 8.8 U and a specific activity of about 0.44 U/mg of protein.

3.3. Characterization of purified recombinant chitinase

Effect of temperature and pH on the activity and stability of purified enzyme.

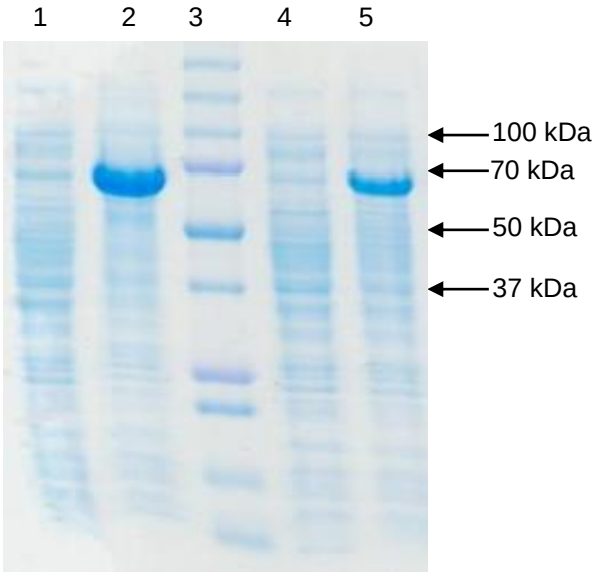

Figure 1. SDS-PAGE of chitinase expressed in *E. coli* T7 at 18°C and 25°C

Note: 1. Non-induced 18°C; 2. Induced 18°C by 0.4mM IPTG at OD 0.5; 3. Ladder; 4. Non-induced 25°C; 5. Induced 25°C by 0.4mM IPTG at OD 0.5

Table 1. Chitinase activity in *E. coli* T7 induced by the addition of 0.4 mM IPTG at OD 0.4 - 0.5. Chitinase activity was measured using pNP-$(GlcNAc)_2$ as substrate.

Temperature of expression	Non-induced/induced by IPTG 0.4mM	Specific activity (U/mg)	Volumetric activity (U/L)
18°C	Non-induced	0.020	0.97
	Induced	0.240	12.90
25°C	Non-induced	0.002	0.19
	Induced	0.120	7.20

Table 2. Purification of recombinant chitinase from *B. licheniformis* DSM13 in *E. coli* T7 at 18°C (from 1 L of LB culture broth)

Purification steps	Total Activity[(a)] (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	12.9	54	0.24	
Purified enzyme	8.8	20	0.44	68.2

Note: [a]pNP-$(GlcNAc)_2$ was used to determine enzyme activity

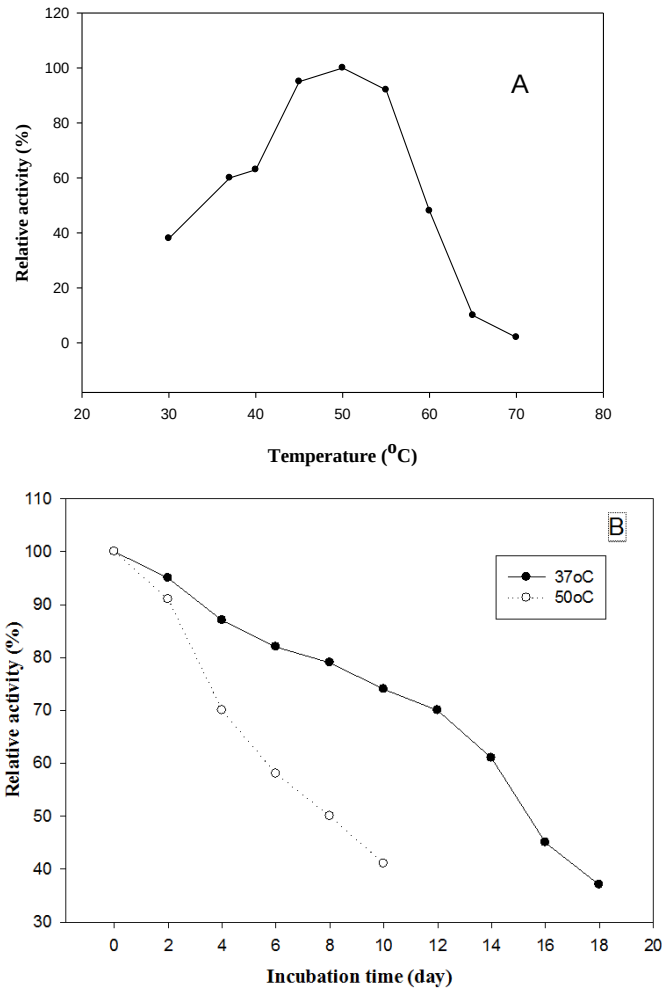

Figure.2. Effect of temperature on activity (A) and stability (B) of recombinant chitinase from *B. licheniformis* DSM13

The optimal temperature of the purified recombinant chitinase was 50°C when using 2% colloidal chitin as a substrate for a 30-min assay (Figure 2A). After 8 days of incubation at 37°C and 50°C, the enzyme still remained 80 and 50% of initial activities, respectively (Figure 2B). The results showed that the enzyme has good thermostability (half-lives of 15 days and 8 days at 37°C and 50° C, respectively), and good stability in the pH range from pH 5 to pH 9.

Figure 3 indicates that the pH optimum of recombinant chitinase is in the range of 7 - 8 and highest at pH 7.5 (Figure 3A). The enzyme was very stable at pH 6, 7, and 8 (remained over 60% of activity after 15 days of incubation).

These results were comparable with that described by Nguyen *et al.* (2012), in which the chitinase gene was cloned and expressed in the expression system of *Lactobacillus plantarum*. The characteristics of the recombinant chitinase were not significantly different when expressed in *E. coli* or *L. plantarum*.

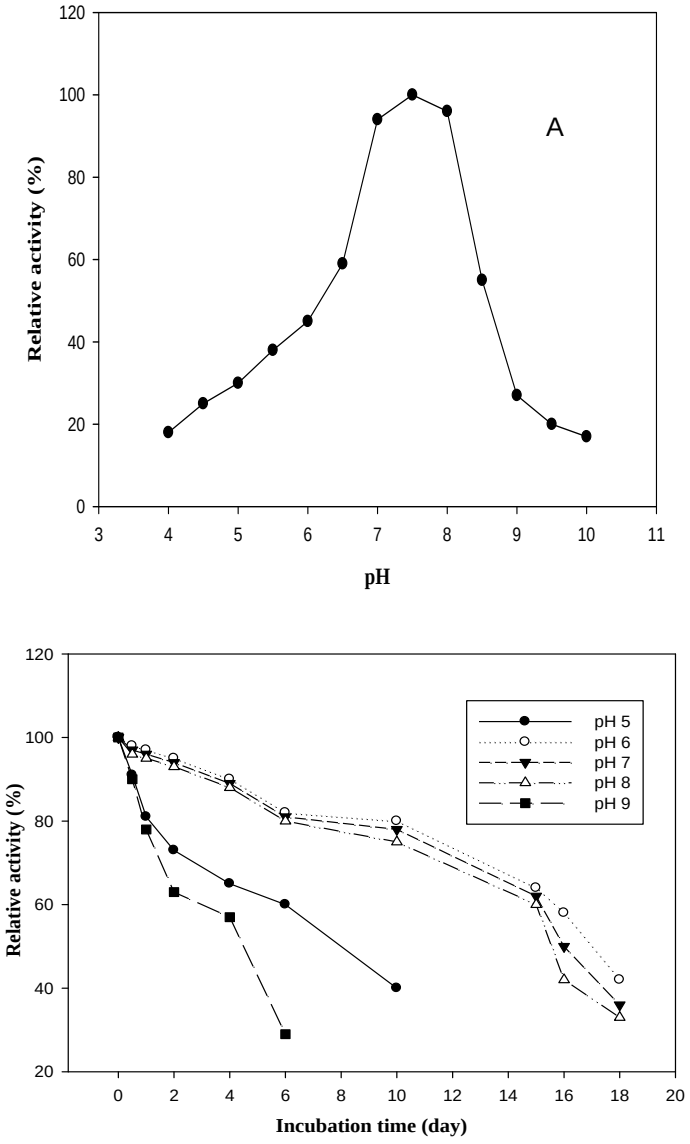

Figure.3. Effect of pH on activity (A) and stability (B) of recombinant chitinase from *B. licheniformis* DSM13

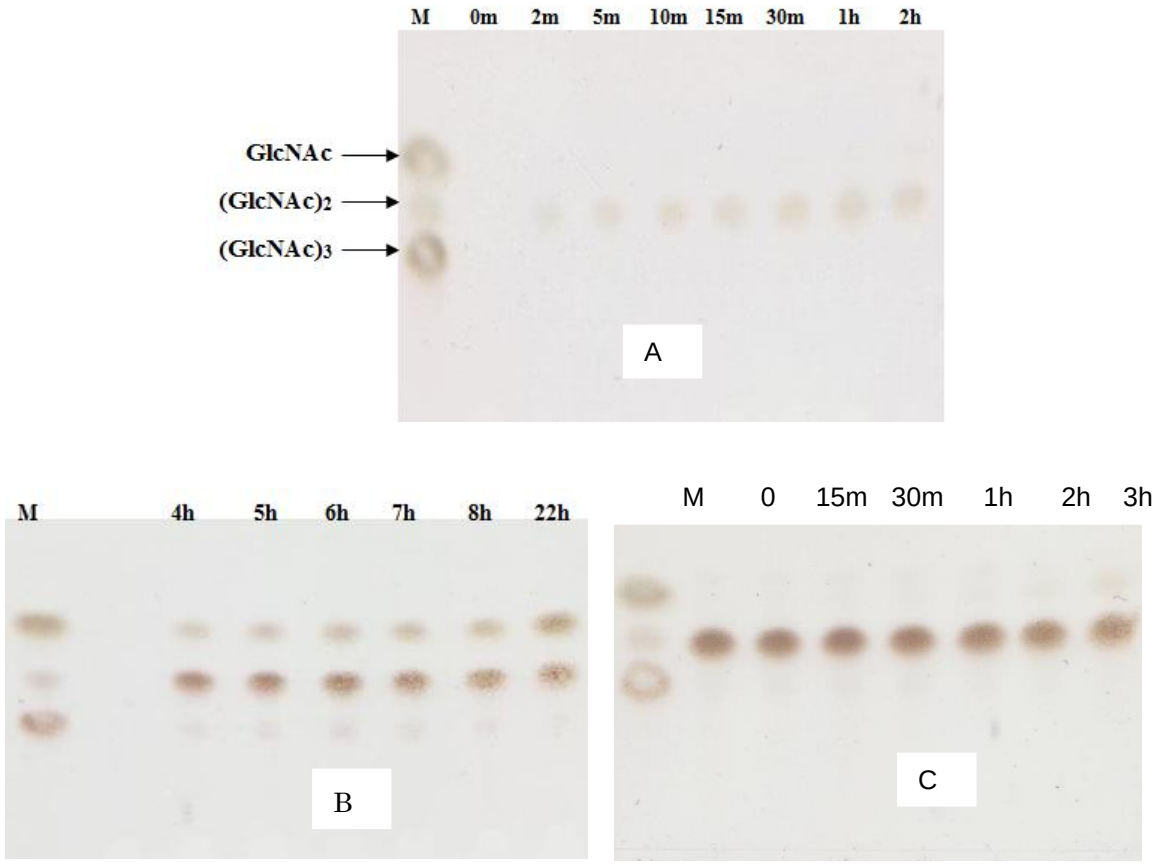

Figure 4. Thin layer chromatography showing colloidal chitin hydrolysis products of purified recombinant chitinase using 2% colloidal (A), 20% coloidal chitin (B), and 10 mM diacetyl chitobiose (C) as substrates, M: a standard mixture of GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$

Analysis of hydrolysis products using TLC

The products of the chitinase-catalyzed hydrolysis of colloidal chitin (2% and 20%) and di-acetyl chitobiose were studied by thin layer chromatography. The main hydrolysis product of colloidal chitin was diacetyl chitobiose (Figures 4A and 4B), while pure di-acetyl chitobiose apparently was not hydrolyzed within 8 h under the conditions selected (Fig. 4C). This result was quite similar to that studied by Nguyen *et al.* (2012).

4. CONCLUSIONS

Recombinant chitinase activity expressed in *E. coli* T7 using pET-21d was approximately 8 times higher than previously reported in *L. plantarum*. The characteristics of recombinant enzymes expressed by these two systems were almost identical. The recombinant enzyme was stable at both 37°C and 50° C, and in the pH range of 5 - 9. The main hydrolysis product was di-acetyl chitobiose. The recombinant chitin from *B. licheniformis* DSM 13 expressed in *E. coli* T7 is promising for the production of functional diacetyl-chitooligosaccharides from chitin-rich aquaculture byproducts.

Acknowledgements

The author is grateful to the Laboratory of Food Biotechnology, Institute of Food Technology, University of Natural Resourses and Life Science, Vienna, Austria for their kind hospitality and support of materials to properly implement this study.

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EFFECT OF DROUGHT STRESS ON PORPHYRIN BIOSYNTHESIS IN RICE SEEDLINGS

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Received date: 15.03.2017 Accepted date: 17.07.2017

ABSTRACT

Porphyrins play vital roles in various biological processes. This study focused on porphyrin biosynthesis control under drought stress in rice plant. The results showed that in response to drought treatment by water withholding, the level of porphyrin intermediates greatly decreased after 36 h of treatment whereas final products (chlorophyll and heme) content just slightly reduced after 60 h of drought stress. The activity of some key enzymes in porphyrin pathway, including ALA-synthesizing capacity, PPO activity, Mg-chelatase activity, Fe-chelatase activity reduced together with the down-regulation of porphyrin biosynthetic genes and nuclear-encoded photosynthetic genes, especially at 48 and 60 h after treatment. It indicated a sensitivity of porphyrin pathway to drought stress. And it also demonstrated a tight control of porphyrin biosynthesis in order to prevent the accumulation of toxic metabolic intermediates by down-regulation of their biosynthesis under drought condition.

Keywords: Chlorophyll, drought stress, heme, porphyrin biosynthesis, rice plant.

Abbreviates: ALA: 5-aminolevulinic acid, *ALAD*: ALA dehydratase, *CHLD*: D-subunit of Mg-chelatase, *CHLH*: H-subunit of Mg-chelatase, *CHLI*: I-subunit of Mg-chelatase, *FC*: ferrochelatase, *GSA:* glutamate 1-semialdehyde aminotransferase, *HEMA*: glutamyl-tRNA reductase, MgProto: Mg-protoporphyrin IX; MgProto ME: Mg-protoporphyrin IX monomethylester; Pchlide: protochlorophyllide, *PORB*: protochlorophyllide oxidoreductase B, *PPO*: protoporphyrinogen oxidase, Proto IX: protoporphyrin IX, ROS: reactive oxygen species.

Ảnh hưởng của hạn tới sinh tổng hợp porphyrin ở cây lúa

TÓM TẮT

Quá trình sinh tổng hợp porphyrin đóng vai trò quan trọng trong các hoạt động trao đổi chất diễn ra trong cơ thể sinh vật. Nghiên cứu của chúng tôi tập trung vào quá trình sinh tổng hợp porphyrin trong điều kiện hạn trên cây lúa. Kết quả cho thấy hàm lượng các chất trung gian trong quá trình sinh tổng hợp porphyrin giảm mạnh sau 36 h ngừng tưới nước, trong khi đó hàm lượng hai sản phẩm cuối của quá trình sinh tổng hợp porphyrin là diệp lục và heme thì chỉ giảm ít sau 60 h xử lý hạn. Hoạt động của các enzyme chìa khóa trong con đường này bao gồm khả năng sinh tổng hợp ALA, hoạt động của PPO, Mg-chelatase, Fe-chelatase cũng giảm cùng với sự giảm biểu hiện của các gen trong quá trình sinh tổng hợp porphyrin và một số gen liên quan tới hoạt động quang hợp được mã hóa trong nhân, đặc biệt ở 48 và 60 h sau khi xử lý hạn. Từ những kết quả thu được cho thấy quá trình sinh tổng hợp porphyrin bị ảnh hưởng rất lớn trong điều kiện hạn. Kết quả nghiên cứu cũng chứng tỏ rằng có một cơ chế phối hợp điều khiển chặt chẽ để ngăn ngừa sự tích lũy các sản phẩm trung gian trong quá trình sinh tổng hợp porphyrin trong điều kiện hạn.

Từ khóa: Diệp lục, Cây lúa, Heme, Khô hạn, Sinh tổng hợp porphyrin.

1. INTRODUCTION

Porphyrins have important function in various biological processes, such as photosynthesis, respiration, morphogenesis, and detoxification. Higher plants produce four classes of porphyrins which consist of chlorophyll and heme (Fig. 1). The porphyrin biosynthetic pathway is divided into four main parts: (1) the formation of 5-aminolevulinic acid

(ALA), (2) the formation of protoporphyrin IX (Proto IX) from eight molecules of ALA, (3) the magnesium porphyrin (Mg-porphyrin) branch to chlorophyll, and (4) the heme-synthesizing branch (Papenbrock and Grimm, 2001; Tanaka and Tanaka, 2007) (Fig. 1).

The biosynthesis of porphyrin is tightly regulated at several levels to coordinate apoprotein synthesis and to avoid the accumulation of porphyrin intermediates (Papenbrock and Grimm, 2001). Plants suffer severe photodynamic damage if these control mechanisms are circumvented. All chlorophyll precursors are potent photosensitizers, so their accumulations will interact with molecular oxygen in the presence of light to produce ROS, such as singlet oxygen and hydrogen peroxide, which cause damage to proteins, lipids, carbohydrates, pigments, and DNA and ultimately result in cell death (Kim *et al.,* 2014; Phung and Jung, 2014 & 2015). Porphyrin intermediate biosynthesis may provide signals to control expression of nuclear genes in response to metabolic activity in chloroplasts (Chi *et al*., 2013). Several studies have been performed to investigate the impact of water stress on photosynthesis (Galmés *et al*., 2007; Massacci *et al*., 2008), however, there were little known about porphyrin biosynthesis in response to drought stress. In our study, important products, key enzymes and related genes in porphyrin biosynthetic pathway were investigated after withholding irrigation to reveal the metabolic regulation of the porphyrin biosynthetic pathway under drought condition.

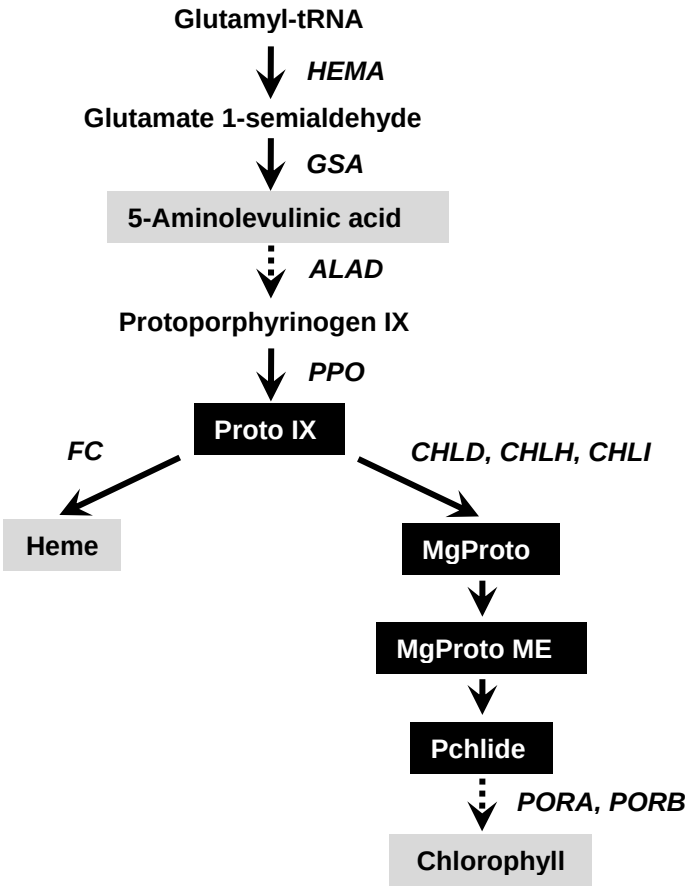

Fig. 1. The tetrapyrrole pathway in plants showing intermediates and genes analyzed in this study

Note: Intermediates quantified in this study are highlighted. Intermediates: Proto IX, MgProto, MgProto ME, Pchlide. Genes and enzymes that correspond to the gene names: HEMA, GSA, ALAD, PPO, FC, CHLD, CHLH, CHLI, PORA, PORB.

2. MATERIALS AND METHODS

2.1. Materials

Rice seedlings (*Oryza sativa* cv. Dongjin fromKorea) were used in this study. They were grown in growth chamber maintained at day/night temperatures of 28°C/25°C under a 14-h-light/10-h-dark cycle (7:00 AM-9:00 PM) with 200 mmol m^{-2} s^{-1} photosynthetic photon flux density.

2.2. Methods

- Drought treatment: Three-week-old rice seedlings were exposed to drought by withholding water for 60 h. Youngest, fully expanded leaf tissues were sampled at 36 h (9:00 AM), 48 h (9:00 PM), 60 h (9:00 AM) after drought treatment. Control plants with sufficient water supply were harvested at the same time as the drought-treated plants for 36 h as previously described (Phung Thi Thu Ha, 2014).

- Determination of porphyrin: For measurement of porphyrin content, plant tissue was extracted then separated and measured by HPLC following the method of Lermontova and Grimm (2006).

- Determination of heme: Heme was extracted and separated by HPLC as described previously (Schneegurt and Beale, 1986).

- *ALA-synthesizing capacity*: ALA-synthesizing capacity was measured using spectrophotometer as described by Papenbrock *et al.* (1999).

- Assays for enzyme activities of tetrapyrrole biosynthesis: The PPO activity was determined using the method of Lermontova and Grimm (2000). Mg-chelatase was assayed as described by Lee *et al.* (1992). Fe-chelatase activity was measured using the protocol from Papenbrock *et al.* (1999).

- *RNA extraction and RT-PCR:* Total RNA was prepared from leaf tissues using TRIZOL Reagent (Invitrogen), and 5 mg of RNA from each sample was used for the reverse transcription reaction (SuperScript III First-Strand Synthesis System; Invitrogen). Subsequently, 50 ng of cDNA was used for RT-PCR analysis. The specific primers were designed based on gene bank database as described previously by Phung *et al.* (2011). Actin was used as the internal control.

Data were analyzed by Microsoft Excel. The data represent the mean ± SE of six replicates from two independent experiments.

3. RESULTS AND DISCUSSION

3.1. Effect of drought on porphyrin intermediates and end products

Rice seedlings were drought treated by withholding irrigation for 60 h. The drought symptom expressed as leaves rolling after 36, 48 and 60 h of treatment. At 60 h after drought treatment, the dehydration symptom was more severe; the leaves lost more than 60% of water content (Fig. 2) as reported in previous studies (Phung Thi Thu Ha, 2014).

To study the effect of drought stress on porphyrin metabolic flux, we first monitored the concentration of Proto XI, the common precursor of both heme and chlorophyll branches, and three intermediates of chlorophyll branch (MgProto, MgProto ME, and Pchlide) before and after withholding water for 36, 48 and 60 h. The result showed that content of all porphyrin intermediates greatly decreased after drought stress in leaves of the rice seedlings. These levels significantly decreased at 36 h of drought treatment although treated plants did not yet exhibit any drought symptom. At 60 h after withholding irrigation, the levels remained in small amount, even Proto IX almost disappeared from the leaves of treated plants (Fig. 3). The decrease of the porphyrin intermediates was directly proportional to the accumulation of H2O2 and malondialdehyde (Fig. 3).

Among the end products of porphyrin pathway, chlorophyll is an important pigment in photosynthetic system of photosynthetic organisms and heme is an essential molecule that is responsible for crucial biological activities including oxygen metabolism and transfer, electron transfer and secondary metabolism (Tanaka and Tanaka, 2007). In porphyrin biosynthetic pathway, drought stress

did not much affect chlorophyll content, whereas heme content slightly reduced in the leaves of treated seedlings (Fig. 3). Taken together with the reduction of porphyrin intermediates (Fig. 3), it indicated that drought stress affected porphyrin intermediates more than the end products. The similar tendency was found in plants responding to chilling, heat and salt stress in previous publication. Phung and Jung (2015) reported that the content of porphyrin intermedieates (Proto IX, MgProto, MgProto ME and Pchlide) drastically declined whereas chlorophyll content slightly decreased only in rice plants under chilling and heat stress. And salt stress led to the reduction in content of Proto IX and MgProto ME but did not affect chlorophyll content (Yun *et al.*, 2012). The excess of porphyrin photosensitizers also caused the accumulation of ROS which damage plant cells (Kim *et al.*, 2014; Phung and Jung, 2014 & 2015), so the degradation dynamics of these photosensitizing porphyrin may lead to reduced ROS production and altered redox state of plastids (Phung *et at.,* 2011) in order to protect plants from drought damage. It may be an additional protective mechanism of plants in response to stress.

3.2. Effect of drought on the expression of nuclear-encoded photosynthetic genes

Retrograde signaling is a process in which plant organelles emit signals that regulate the expression of nuclear genes. The chlorophyll intermediate MgProto as one of such signal acts as a negative regulator of photosynthetic gene expression (Nott *et al.*, 2006). The expression levels of nucleus-encoded photosynthetic genes (*Lhcb1*, *Lhcb6* and *RcbS*) were determined to evaluate their relationship with MgProto under drought stress. The results showed that transcription levels of *Lhcb1* and *Lhcb6*, the genes encoding Lhcb protein of Photosystem II, were down-regulated under water deficit with an earlier decline of *Lhcb6* than *Lhcb1*. The expression of *rcbS* gene encoding the small subunit of Rubisco, also drastically reduced after 48 h of withholding water (Fig. 4). The reduction of MgProto content together with a down-regulation of *Lhcb1*, *Lhcb6* and *RbcS* genes was aslo found in oxyfluorfen- and ALA- treated plants after two days (Phung and Jung, 2014). By contrast, MgProto accumulation led to a down-regulation of *Lhcb* and other nucleus-encoded photosynthetic genes under norflurazon treatment (Strand *et al.*, 2003). Our data indicated that down-regulation of nuclear-encoded photosynthetic genes not due to the accumulation of MgProto but also plant cell damage and accumulation of ROS in rice seedling after stopping irrigation and it also lead to reduced efficiency of photosystem II (Fig. 3 & 4).

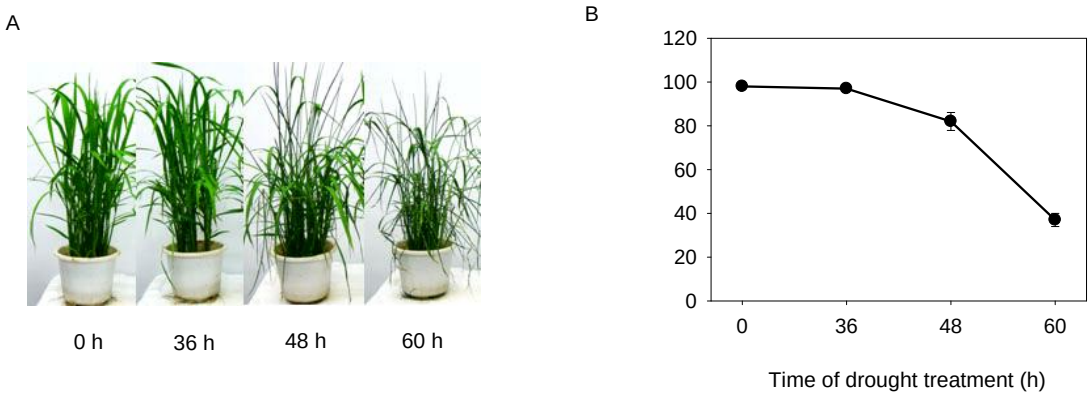

Fig. 2. (A) Phenotypes of rice seedlings and (B) relative water content (RWC) of leaves before and after water withholding for 36 h, 48 h and 60 h

Source: Phung Thi Thu Ha, 2014

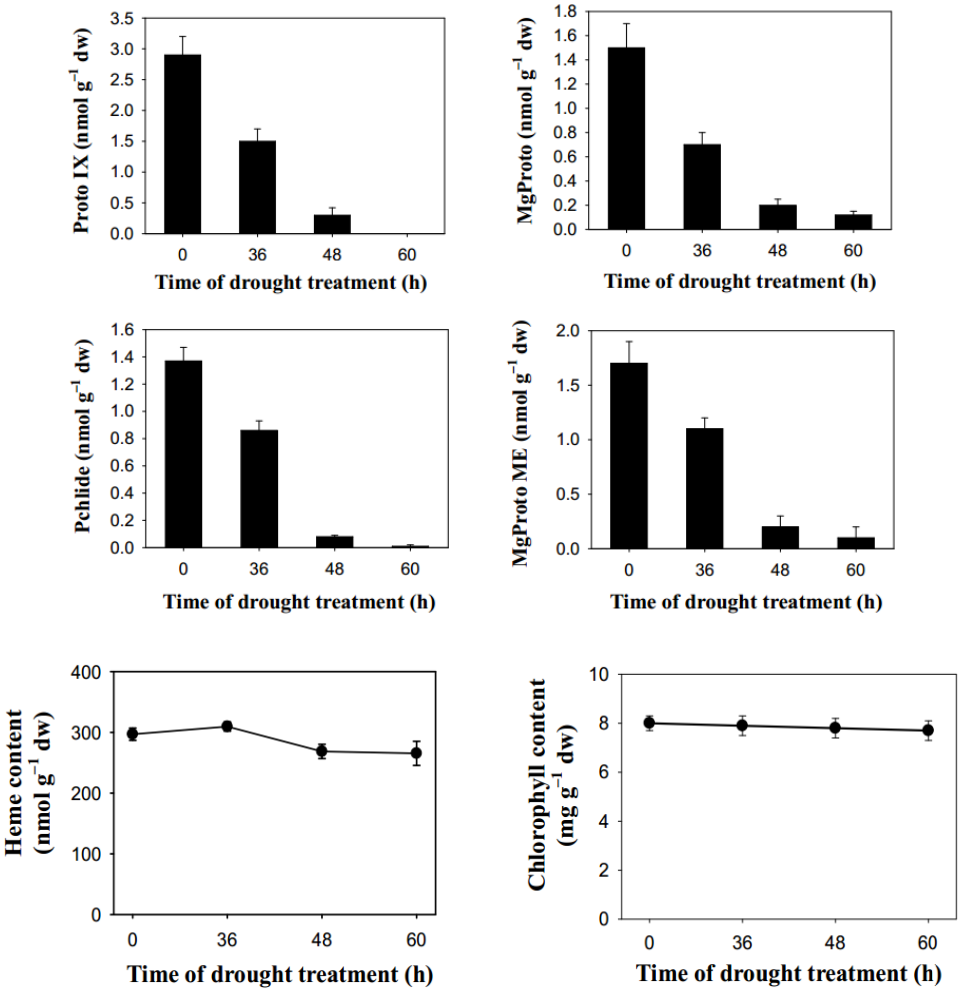

Fig. 3. Effect of drought on tetraphyrrole metabolites flux in rice seedling

Note: The plants were subjected to the same treatments as in Fig. 2. Treatment notations are the same as in Fig. 2. Values are means ± SE of six replicates from two independent experiments

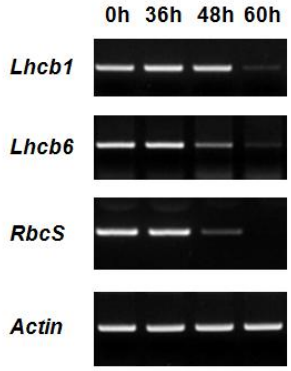

Fig. 4. The expression of nuclear-encoded photosynthetic genes in drought-stressed leaves

Note: Expression analysis of nuclear-encoded photosynthetic genes by RT-PCR. Total RNAs were purified from plants and reverse-transcribed. The resultant cDNAs were used as templates for RT-PCR using Actin as an internal control. The plants were subjected to the same treatments as in Figure 2.

3.3. Effect of drought on enzyme activities of porphyrin biosynthetic pathway and gene expression

In the subsequent study, the activity of some key enzymes in porphyrin biosynthetic pathway was examined. The first step is the corporation of some enzymes in the early of porphyrin pathway scheme to produce ALA, the important precursor of this pathway. ALA synthesizing capacity decreased in rice seedlings in response to drought stress from 48 h after withholding water (Fig. 5). The data correlated with the expression of three corresponding genes *HEMA1*, *GSA* and *ALAD* (Fig. 6). Jain *et al.* (2013) also reported that ALAD activity reduced in leaves of etiolated maize seedlings under water deficit induced by PEG-6000 which led to reduction of ALA content, the first important precursor of porphyrin pathway.

The second enzyme monitored was PPO activity. PPO enzyme catalyzes the formation of Proto IX from Protogen IX. PPO activity declined in rice plants after 36 h of treatment (Fig. 5). It correlated with the reduction of *PPO* gene expression in response to water deficit (Fig. 6).

Mg-chelatase and Fe-Chelatase are two first enzymes of chlorophyll and heme branch, respectively. Both use Proto IX produced by PPO enzyme as a substrate. From Proto IX, Fe-chelatase converts to heme and Mg-chelatase to produce chlorophyll, the end products of these branches. Their activities decreased in treated plants in response to drought. However, Mg-chelatase activity exhibited more drastic decline than Fe-chelatase activity as well as PPO activity and ALA synthesizing capacity (Fig. 5). The expression of chlorophyll branch genes (*CHLH*, *CHLI*, *CHLD*, *PORA*, *PORB*) and heme branch gene (*FC2*) were also down-regulated in rice plants after drought treatment (Fig. 6). Those data indicated that drought stress affected chlorophyll branch more than heme branch. This might relate to the role of heme in detoxification system.

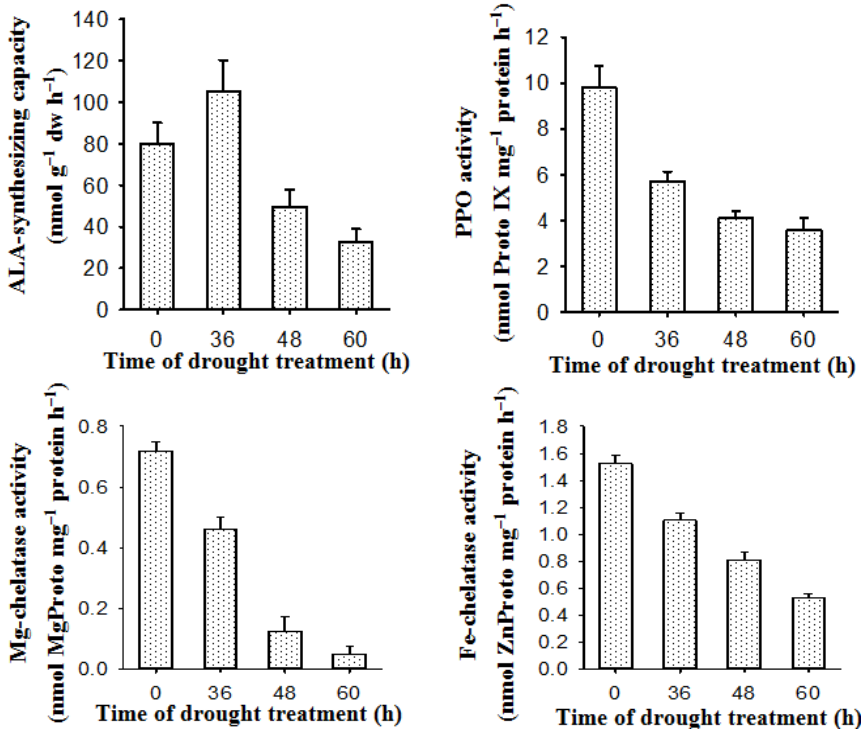

Fig. 5. Effect of drought on porphyrin synthesizing enzyme activity in leaves of rice seedling

Note: The plants were subjected to the same treatments as in Fig. 2. Treatment notations are the same as in Fig. 2. Values are means ± SE of six replicates from two independent experiments

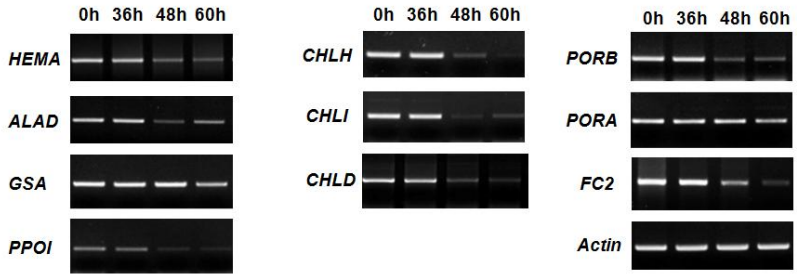

Fig. 6. Drought-induced changes in expression of genes encoding the porphyrin pathway enzymes

Note: Total RNAs were purified from plants and reverse-transcribed. The resultant cDNAs were used as templates for RT-PCR using Actin as an internal control. The plants were subjected to the same treatments as in Figure 2. Although activity of Mg-chelatase, expression of chlorophyll branch genes and chlorophyll intermediates decreased under drought, chlorophyll content slightly decreased only (Fig 3, 5, 6). It is in agreement with Jain et al. *(2013) that water deficit affects chlorophyll formation rather than its degradation. Down-regulation of the porphyrin biosynthesis genes and enzyme activity have also been shown in chilling-stressed seedlins (Mohanty* et al.*, 2006) and in oxyfluorfen - and ALA- treated rice plants (Phung and Jung, 2014).*

Our data indicated a sensitivity of porphyrin pathway to drought stress. It also demonstrated a tight control of porphyrin biosynthesis in order to prevent the accumulation of toxic metabolic intermediates by down-regulation of their biosynthesis under drought condition.

4. CONCLUSION

In this study, rice plants which were drought treated by withholding irrigation led to the down-regulation of porphyrin metabolite flux (including porphyrin intermediates and the end products, the activity of key enzymes and the expression of porphyrin biosynthetic genes) in order to prevent the accumulation of harmful singlet oxygen generating porphyrins. It also caused the reduced expression of nuclear-encoded photosynthesis genes (*Lhcb1*, *Lhcb6* and *RbcS*) as a result of cellular damage by ROS accumulation.

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A MYXOBACTERIUM STRAIN ISOLATED IN VIETNAM PRODUCES EREMOPHILENE-LIKE SESQUITERPENE

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Received date: 10.09.2017 Accepted date: 10.10.2017

ABSTRACT

Sesquiterpenes, a class of terpenes, consist of three isoprene units, constituting a highly diverse class of natural bioactive compounds that are found principally in plants but also in fungi and some invertebrates and myxobacteria as well. In this study, by using different approaches for isolation, analysis of morphology and bio-physio-chemical properties and comparison of the sequences of 16S ribosomal RNA genes, three strains belonging to the group of myxobacteria were isolated from soil samples collected from some areas in Vietnam. The sesquiterpene biosynthesis in the isolated strains was investigated. The data showed that the isolated strain DL1 was able to form fruiting body and sysnthesize an eremophilene-like sesquiterpene.

Keywords: 16S rRNA, eremophilene, Myxobacteria, sesquiterpene, secondary metabolites.

Phân lập và phát hiện chủng vi khuẩn Myxobacteria ở Việt Nam có khả năng tổng hợp Sesquiterpene tương tự Eremophilene

TÓM TẮT

Sesquiterpene được tìm thấy chủ yếu ở thực vật, nấm và một số động vật không xương sống. Gần đây, sinh tổng tổng hợp sesquiterpene ở một số vi khuẩn thuộc nhóm myxobacteria đã được phát hiện. Myxobacteria là một nhóm vi khuẩn tiềm năng được nhiều nhóm nghiên cứu trên thế giới quan tâm nhằm khai thác các hợp chất thứ cấp mới ứng dụng trong y dược. Tuy nhiên cho đến nay ở Việt Nam hầu như chưa có nghiên cứu về phân lập và xác định đặc điểm của các vi khuẩn này. Trong nghiên cứu này, bằng cách sử dụng các kỹ thuật phân lập, quan sát mô tả hình thái, đặc điểm sinh lý hóa và so sánh trình tự 16S rRNA, 3 chủng vi khuẩn thuộc nhóm myxobacteria đã được phân lập từ các mẫu đất thu thập ở nhiều địa phương khác nhau ở Việt Nam. Khảo sát khả năng tổng hợp sesquiterpene từ các mẫu vi khuẩn phân lập cho thấy chủng DL1 có khả năng sinh thể quả và tổng hợp một sesquiterpene giống như eremophilene.

Từ khóa: 16S rRNA, Eremophilene, hợp chất tự nhiên, Myxobacteria, Sesquiterpene.

1. INTRODUCTION

Sesquiterpenes constitute a highly diverse class of natural bioactive compounds for medicine, flavors and fragrance ingredients, food additives, and agrochemicals (Bártíková *et al.*, 2014). So far, sesquiterpenes have been reported as secondary metabolites synthesized mainly in higher plants, fungi, and some invertebrates. Recently, some research groups have found that the biosynthesis of sesquiterpenes occurs in some bacteria, notably, myxobacteria. These bacteria have been reported as an excellent natural sources for the discovery of new polyketides for wide-range application (Wenzel and Müller, 2007).

Myxobacteria are large groups of bacteria living predominantly in the soil. These bacteria

do not have flagella and move on solid surfaces by gliding (Schneiker *et al.,* 2007). Of proteobacteria, only myxobacteria belonging to delta protobacteria (δ-proteobacteria) are able to develop fruiting body under unfavorable conditions including pH, temperature or lack of nutrient. The spores (myxospores) are separated or grouped together to form fruiting body that can appear on the surface of the medium (observed in the genera *Melittangium and Stigmatella*) or in the medium (observed in the genera *Angiococcus, Polyangium, Cystobacter, and Sorangium)* (Shimkets *et al.*, 2006). The fruiting bodies of myxobacteria vary from species to species with different size, shape and colour (Shimkets *et al.*, 2006). The life cycle of myxobacteria is quite complicated because of the change of morphology in different growth stages including vegetative stage, myxospore and fruiting body. The ability to form fruiting body is one of the easiest sign to be recognized and it is also one of the interesting characteristics of myxobacteria.

So far, more than 100 different basic compounds and approximately 500 structural variants have been isolated from myxobacteria (Reichenbach,1999; Gerth *et al.*, 2003). About 7.500 different myxobacteria have been isolated and analyzed chemically (Gerth *et al.*, 2003; Wenzel and Müller, 2007). In the large group of myxobacteria, the genus *Sorangium* produces nearly 50% of total metabolites isolated from myxobacteria (Gerth *et al.*, 2003; Schneiker *et al.*, 2007). With a high potential of application, isolation and characterization of new strains of myxobacteria are of significance.

In this study, soil samples collected from different areas of Vietnam were used for isolation of myxobacteria. Analysis of morphology, characterization of bio-physio-chemical properties and comparison of the sequences of the 16S ribosomal RNA gene from isolated strains were carried out. Sesquiterpenes released from culture media of these strains were also analyzed.

2. MATERIALS AND METHODS

2.1. Collecting soil samples

Soil samples collected from different areas in Vietnam, i.e.Da Lat, Bac Giang, Gia Lam, Kon Tum, Ninh Binh, Soc Son, Son Tay, Thai Nguyen, Tuyen Quang, Vinh Phuc, Yen Bai were used to isolate myxobacteria. Collected soil samples were dried and stored in closed plastic bags at 25 to 30°C and moisture from 75 to 85%.

2.2. Isolation media and antibiotics

For isolation of myxobacteria, several media ST21, ST21CX, VY/2, WAT, EBS and LB were used (Reichenbach and Dworkin, 2001). The components of the ST21 medium included macro elements (g/L): K_2HPO_4 1, yeast extract 0.02, KNO_3 1, $MgSO_4.7H_2O$ 1, $CaCl_2.2H_2O$ 1, $FeCl_3$ 1, $MnSO_4.7H_2O$ 0.1, agar 10, and microelements (mg/L): $MnCl_2.4H_2O$ 0.1, $CoCl_2$ 0.02, CuSO4 0.01, $Na_2MoO_4.2H_2O$ 0.01, $ZnCl_2$ 0.02, LiCl 0.005, $SnCl_2.2H_2O$ 0.005, H_3BO_3 0.01, KBr 0.02, EDTA Na-Fe^{3+} 0.008. The ST21CX medium was modified from ST21 by adding 25 µg/ml cycloheximide. The medium VY/2 included (g/L): Baker's yeast 5, $CaCl_2.2H_2O$ 1, cyanocobalamin 0.5,agar 15**.**The WAT medium included: $CaCl_2.2H_2O$ 1.1% (w/v), agar 1.5% (w/v). The medium EBS included (w/v in percent): peptone from casein 0.5, proteosepeptone 0.5, peptone from meat 0.1, yeast extract 0.1, pH 7.0. The LB medium included (w/v in percent): peptone 1, yeast extract 0.5, NaCl 1, pH 7.5. Several antibiotics including cyclohexamide (CX), nystatin (NYS), kanamycin (KA), gentamicin (GEN) were used at 2.5 mg/100 mL.

2.3. Isolation of myxobacteria

The collected soil samples were treated with a combination of two antibiotics CX and KA before isolation. A combination strategy using different methods were applied to isolate myxobacteria including trapping method by rabit dung, filter paper based ST21CX medium, YV/2 medium for the formation of spore and fruiting body, andWAT-*E.coli* based medium for

development of fruiting body (Reichenbach and Dworkin, 2001; Shimkets *et al.*, 2006; Hyun *et al.*, 2008; Li *et al.*, 2013).

2.3.1. Enrichment of myxobacteria by trapping method

The dry soil samples were prepared as a layer with 10-15 mm thick on a petri dish and kept moist by sterile water during the incubation time. Dried rabit dung pellets (about 0.5 cm) were sterilized by autoclaving and put on the dry soil samples and incubated at 30°C (Figure 1). After one week, the dung pellets were transferred to the filter paper based ST21CX medium.

2.3.2. Isolation on the filter paper based ST21CX medium

The filter paper based ST21CX medium used for isolation myxobacterium was previously described by Shimkets *et al.* (2006), Hyun *et al.* (2008), Li *et al.* (2013). Soil samples were spread on a sterile filter paper and plated on the ST21CX medium and incubated at 30°C for 20 to 30 days. The formation of bacterial colonies and fruiting bodies were observed continuously during the incubation period.

2.3.3. Isolation on VY/2 medium

The VY/2 medium used to evaluate the bio-physiological properties of isolated myxobacteria including the formation of spore and fruiting body was previously described by Shimkets *et al.* (2006) and Hyun *et al.* (2008). The development of fruiting bodies on the surface layer of the medium was visualized based on typical sign of myxobacteria. The fruiting bodies on the ST21CX medium were carefully collected and transferred to VY/2 medium to observe gliding of the bacterium and the formation of fruiting bodies. A different combination of antibiotics were applied to avoid contamination for the isolates of myxobacteria.

2.3.4. Isolation on WAT-E. coli based medium

The WAT-E. coli based medium was used for the purification of myxobacteria (Shimkets *et al.*, 2006). *E. coli* obtained from liquid media was sterilized and spread on agar WAT medium to form WAT- *E. coli* based medium. The fruiting bodies of myxobacteria on ST21CX medium were streaked on WAT- *E. coli* based medium to observe further development of the fruiting bodies.

2.3.5. Purification of myxobacteria

Myxobacteria were purified using fruiting bodies obtained from selected media. The purification steps were carried out several times to remove bacteria that may contaminate the gliding colonies. Two alternative methods based on temperature and antibiotics were applied. When the fruiting bodies or spores were formed on selected media, the temperature was increased to 58°C for 10 minutes to eliminate some bacteria (Reichenbach, 1983). In the methods using antibiotics, the fruiting bodies were transferred to EBS medium and shaked at 30°C overnight in the presence of combinations of different antibiotics (CX,KAN, GEN and NYS). This step was carried out several times and the fruiting bodies were then transferred to the VY/2 medium for the formation of the vegetative form of myxobacteria (Reichenbach, 1983).

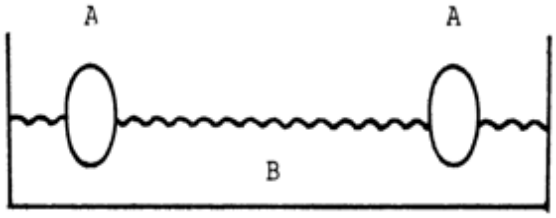

Figure 1. Draw showing the trapping method to enrich myxobacteria

Note: A: Sterilized dried rabbit dung pellets, B: soil sample

2.4. Characterization of bio-physio-chemical properties of isolated bacteria

The cellulase activity of myxobacteria, especially *Sorangium* were observed by the degradation of filter paper on the ST21CX medium in 10, 15, 20, 25, 30 days. The sterile paper was plated on the surface of the medium. The method was applied for the cells in the vegetative stage or gliding form. An agar medium containing cellulose as substrate was also applied to observe the cellulase activity (Reichenbach and Dworkin, 1986). The gram staining was carried out as described (Gram,1884).

2.5. DNA extraction, amplification and analysis of 16S ribosomal RNA

The pure isolated myxobacteria were cultured on liquid ST21CX medium for 4 days at 26ºC with shaking at 200 rpm/min. Cell pellets were collected by centrifuge at 10.000 g for 10 min. The DNA extraction was carried out according to the method described by Wilson (2001). The 16S ribosomal RNA sequence was amplified using primers W4F (GTAAGACAGAGGGTGCAAACGT) and 16SmycoR (GGGTTGCGCTCGTTGCG) (Wu *et al*., 2005; Hyesook *et al*., 2008). The PCR (25 µl) included: 0.5 µl DNA (~100 ng); 0.5 µl W4F (10 pmol), 0.5 µl 16SmycoR (10 pmol); 1.5 µl $MgCl_2$ (25 mM); 2.5 µl 10X PCR buffer; 2 µl dNTP (2.5 mM); 0.25 µl Taq DNA polymerase (5 U/µl). PCR thermal cycle: 95ºC/4 min; 35 cycles (94ºC/1 min; 53°C/1 min; 72ºC /45 min); 72ºC/10 min. PCR product was purified with QIAquick PCR Purification Kit (QIAGEN) and sequenced. The data were analyzed using Bioedit program and the sequences were compared with nucleotide database in GenBank by BLAST at NCBI (www.ncbi.nlm.nih.gov).

2.6. Solid phase micro-extraction- gas chromatography-massspectroscopy (SPME-GC-MS) analysis of volatiles

The isolated strain D1 was grown in ST21CX liquid medium in a headspace flask sealed with septum and aluminium cap. Carboxen-polydimethylsiloxane (CAR/PDMS, 75 µm) fibre with manual holder purchased from Sigma-Aldrich was used for the extraction of volatiles. The volatiles were collected for 24h, and then analyzed by GC/MS as previously described (Ly *et al*, 2017). Volatiles were extracted by exposing the solid phase microextraction (SPME) fibre to the headspace of the culture flask that was maintained at 40°C for 30 min. For thermal desorption, the SPME fibre was quickly inserted into the GC injector. A desorption time of 3 min at 250°C was used in split mode (1:10).

3. RESULTS AND DICUSSION

3.1. Isolation of myxobacteria

As described by Reichenbach (1983), dung of many herbivores such as sheep, goat and rabit is natural substrate sources as effective baits for the isolation of myxobacteria. Many strains of myxobacteria, such as *M. fulvus, M. virescens, Sorangium cellulosum* were also often found in dung of herbivores (Reichenbach,1999). In the present study, rabit dung pellets were utilized to trap myxobacteria (Figure 2).

After 1 week of incubation, rabit dung pellets were transferred to filter paper based ST21CX medium. Some colonies were formed on the filter paper but this process took a long time, up to several weeks (data not shown). Although this method was efficient as reported elsewhere, in this study, however, the amount of fruiting bodies appeared very low and hardly to observe. Therefore, an alternative method was carried out by placing soil samples directly on the surface of the filter paper based ST21X medium (Figure 3A, B, C). Athough various combinations of antibiotics and fungicide such as cyclohexamide, nystatin, kanamycin, and gentamycin were used in the isolation and purification steps, cycloheximide treated soil samples were most efficient because this antibiotic inhibited the protein production of eukaryotic cells including fungi. The data of antibiotic selection and inducing for fruiting bodies are summarized in Table 1. Results

showed that the duration for growth and degradation of filter paper was about 10 days. In some samples, molds were observed after the 10th day of incubation. Due to the ability to degrade filter paper and form fruiting bodies, colonies of myxobacteria are quite easy to select. Several cycles of purification were carried out by a combining formation of fruiting bodies and germination of myxospores from collected fruiting bodies (Figure 3B, D, E).

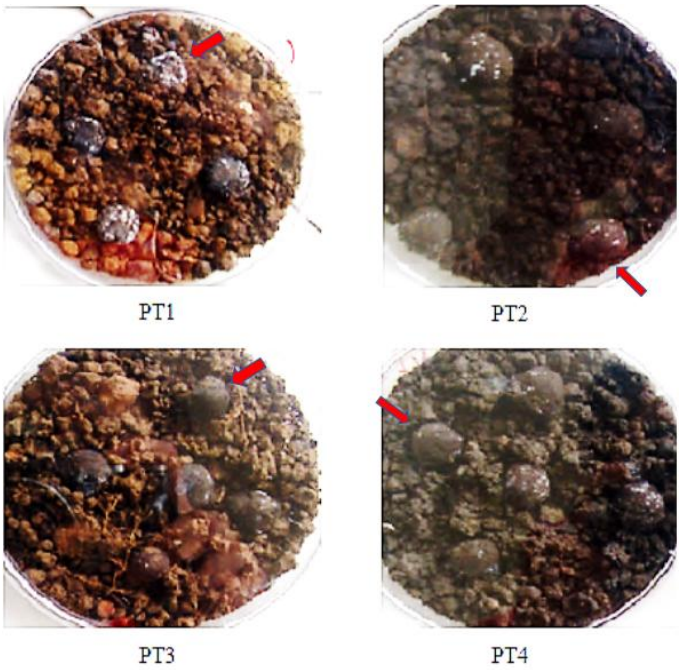

Figure 2. Trapping of myxobacteria by using rabit dung pellets

Note: PT1-2 and PT3-4 are soil samples collected from Gia Lam and Ba Vi, respectively. Arrows indicate the rabit dung

Table 1. Influence of antibiotic treatment on soil samples

Soil sample	Antibiotic treatment				
	CX+ NYS	CX+ KAN	CX+GEN	CX+KAN+NYS	CX+KAN+GEN
DL1	+	+	C	-	-
BG4.1	+	+	-	-	*
BG6.1	+	-	-	-	*
BG6.2	C	C	C	+	-
BG10	-	+	-	*	
GL1	+	+	-	-	-
KT1	-	-	+	*	*
NB5	C	C	C	-	+
SS1	+	C	-	*	*
ST1	C	C	C	-	+
TN1	+	-	-	*	*
TQ7	+	+	-	*	*
VP1	C	C	-	C	+
YB2	+	+	-	*	*

*Note: DL, BG, GL, KT, NB, SS, ST, TN, TQ, VP, and YB were soil samples collected from Da Lat, Bac Giang, Gia Lam, Kon Tum, Ninh Binh, Soc Son, Son Tay, Thai Nguyen, Tuyen Quang, Vinh Phuc, Yen Bai, respectively. The number in dicates the number of collected samples. **C**: contamination, (-) no colony; (+): colony forms fruiting body; (*): colony without forming fruiting body.*

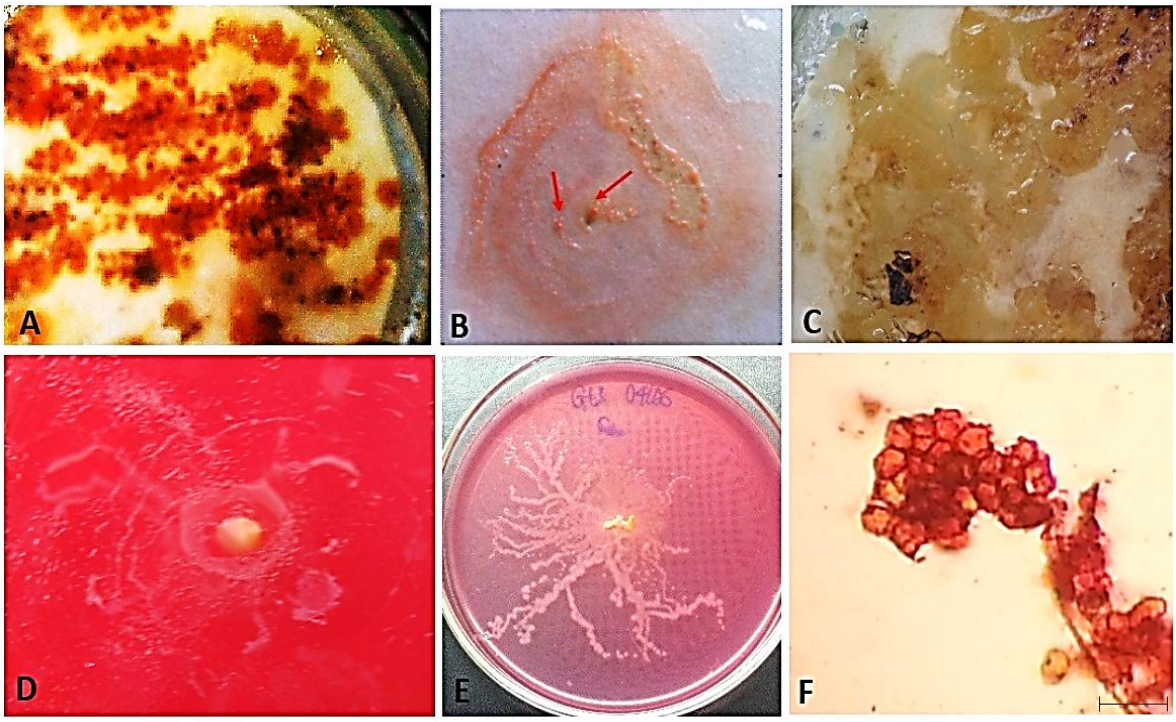

Figure 3. Morphology and formation of fruiting bodies of isolated bacteria

Note: A: Growth and gliding of vegetative form on filter paper based ST21CX medium; ***B****: formation of fruiting bodies, red arrows indicate the fruiting bodies;* ***C****: gliding of bacteria on filter paper based ST21CX medium (vegetative stage);* ***D****,* ***E****: gliding of bacteria on VY/2 medium;* ***F****: an opened fruiting body containing myxospores observed under microscope (gram negative), scale bar indicates 10 µm.*

Several types of colonies with colours were observed on filter paper including white, yellow, orange, pink and brown that spread on the filter papers adjacent to the position of soil samples (Figure 3A, C). This phenomenon was very common in a number of reports (Shimkets *et al.*, 2006). After 20 days of incubation, isolated bacteria from 26 soil samples formed fruiting bodies. Although it has been reported that the fruiting bodies are formed under unfavorable conditions, in this study, the formation of fruiting bodies was observed when there was a lack of nutrients or the surface of the filter paper became dry. Fruiting bodies appeared in some samples from the 20th days of incubation and existed in a short time, from 2 to 3 days. By using gram staining method, the myxospores from the fruiting body of the strain DL1 were observed (Figure 3F). The morphology of the myxospores from strain DL1 was similar to those of *Sorangium cellulosum* (Gerth *et al.*, 2003).

3.2. Sequence comparison and analysis

The isolated DNA samples from purified strains GL2, YB2, DL1, NA2 and TQ7 (Figure 4, left) were used as template for PCR to amplify the 16S ribosomal RNA gene (16S rDNA). Data showed that the size of 16S rDNA was about 600 bp (Figure 4, right). The sequences of the 16S rDNA were obtained by service of 1st BASE sequecining Company (data not shown). Local alignment search tool (BLAST) with sequences of 16S rDNA as queries revealed that the 16S rDNA sequence of the strain GL2 showed the highest similarity (99%) to *Sporocytophaga myxococcoides* DSM 11118 (NR025463) (Figure 5A) and the 16S rDNA sequence of the strain YB2 showed 98% similarity to uncultured bacterium KMS200711-118 (EU881332.1) and 97% similarity to *Myxococcales bacterium* (FJ435064) (Figure 5B). The 16S rDNA sequence of DL1 showed similarity of 99% to *Polyangium* sp. (KC862608) and 98% to *Sorangium cellulosum*

strain X3T8 (HQ623117) (Figure 5C). The 16S rDNA sequences of the strains NA2 and TQ7 were highly similar to those of *Pseudomonas* sp. (data not shown). In general, by the sequence comparison, the isolated bacteria with 97-98% similarity of 16S rRNA were classified into an operational taxonomic unit. All isolated strains belonged to delta-protobacteria. Based on the morphology, bio-physio-chemical properties, the bacterial strains GL2 and YB2 and DL1 samples were classified into myxobacteria and belonged to *Sporocytophaga myxococcoides* GL2, *Myxococcales bacterium* YB2 and *Polyangium* sp. DL1, respectively.

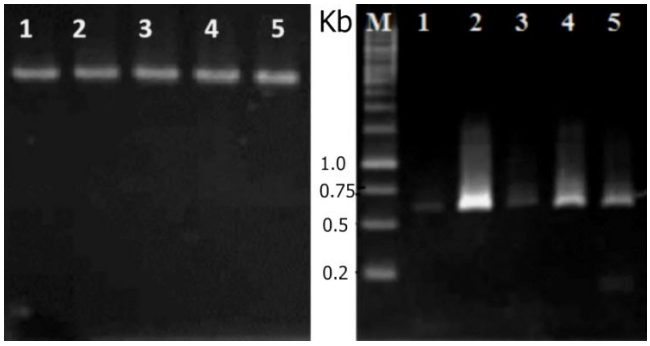

Figure 4. DNA extraction and PCR amplification of 16S ribosomal RNA

Note: Agarose gel electrophoresis of total DNA of samples GL2, YB2, DL1, NA2 and TQ7 (left) and PCR products of 16S ribosomal RNA (right), respectively. M: DNA ladder FluoroBand 1KB (0,25-10kb). The PCR products have size of about 600 bp (0.6 Kb)

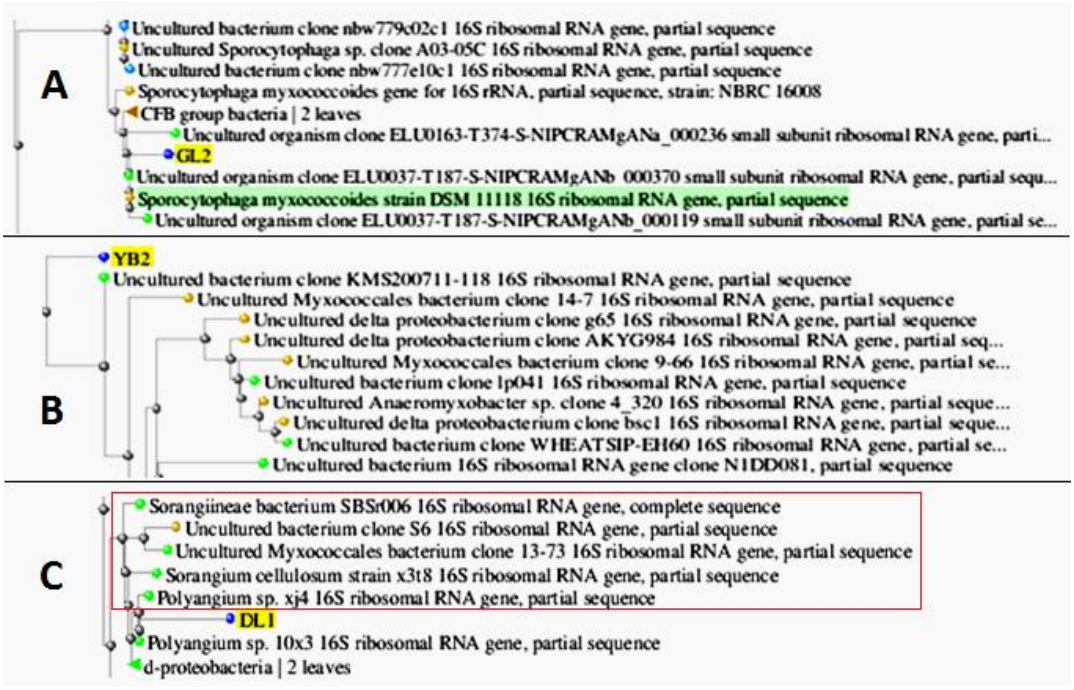

Figure 5. Phylogenetic analysis of 16S rDNA of isolated bacteria

***Note: A**: 16S rDNA of GL2 shows 99% similarity to Sporocytophaga myxococcoides DSM 11118 (NR025463); **B**: 16S rDNA of YB2 shows 97% similarity to Myxococcales bacterium (GenBank accession number: FJ435064); **C**: 16S rDNA of DL1 shows 99% similarity to Polyangium sp. (KC862608) and 98% to Sorangium cellulosum strain X3T8 (HQ623117). All isolated strains belong to delta-protobacteria. The phylogenetic trees (A, B, C) were plot directed from the online BLAST at NCBI. No bootstrap values were displayed.*

3.3. Sesquiterpene detection from volatile by SPME-GC/MS analysis

A solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC/MS) was set up to detect volatile compounds released from the culture. Analyzing the entrapped volatile compounds from the cultures of three isolated strains revealed that in the metabolites of the strain DL1, sesquiterpene was detected with a relatively high content (Figure 6). The relative ion abundances of this compound were similar to those of eremophilene produced by myxobacterium *Sorangium cellulosum Soce56* (Schifrin *et al.*, 2015) for ion fragments: 53, 67, 79, 90, 105, 118, 133,1 47, 161, 189, 204 m/z ions (Figure 7). This suggested that the detected sesquiterpene was likely eremophilene. In addition, GC-MS analysis also revealed several types of volatile compounds including ketones, alcohols, esters, and aromatic compounds (Figure 6A). Several reports showed that eremophilene could be also oxidized to form multiple derivatives which may have cytotoxic, anti-inflammatory and antiviral properties (Schifrin *et al.*, 2015). In terms of ecological function, the sesquiterpene eremophilene may help the myxobacteria to "communicate" or showing social activity to facilite the complex lifestyle such as gliding and forming of fruiting bodies upon starvation or stress condition.

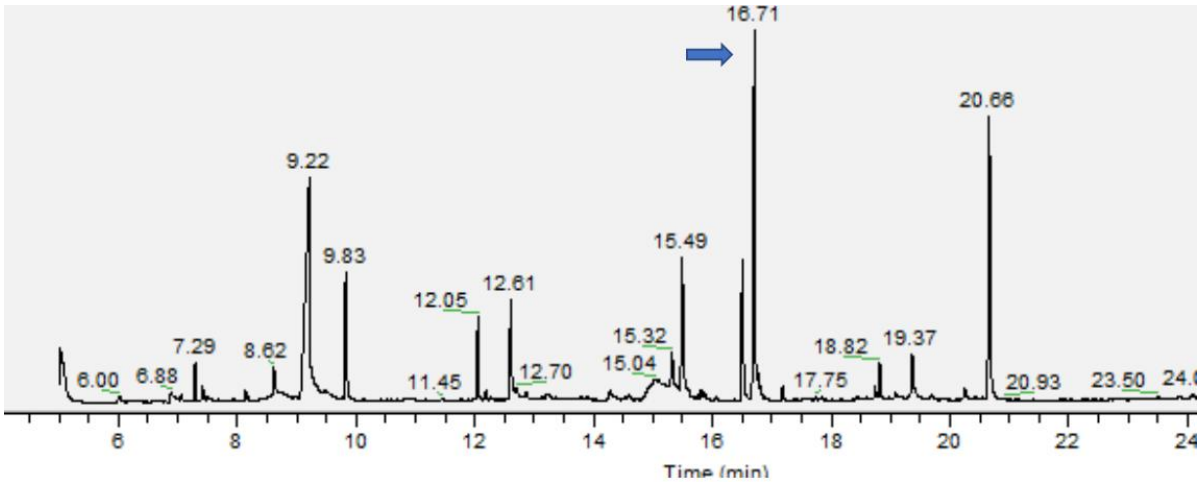

Figure 6. Total ion chromatograms of volatile metabolites

Note: Volatile metabolites collected from the culture medium of the isolatedstrain DL1. The arrow represented the peak with highest content of a sesquiterpene.

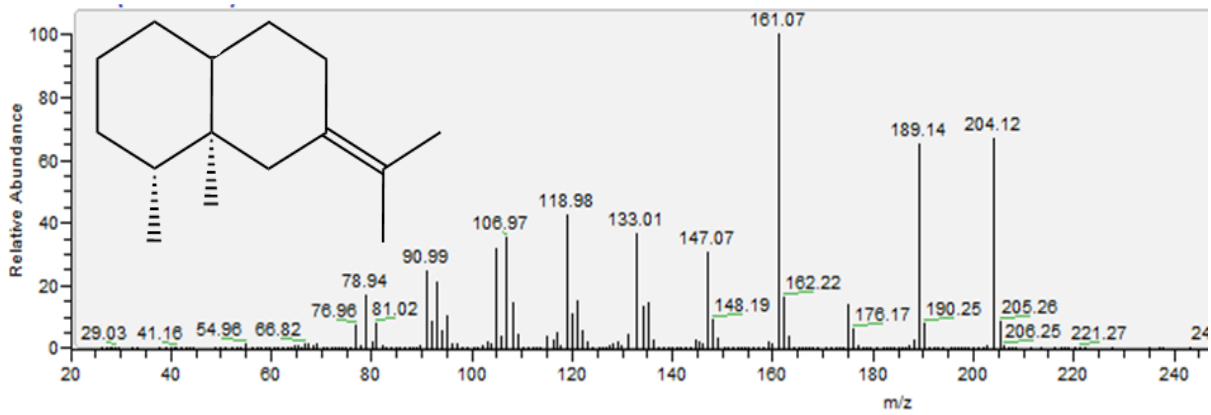

Figure 7. Mass spectra of the detected sesquiterpene

4. CONCLUSION

Study on morphology, bio-physio-chemical properties and phylogenetics of isolated soil bacteria showed that three bacterial strains GL2, YB2, DL1 were likely classified into the group of myxobacteria. These bacterial strains were able to glide and form fruiting bodies on the filter paper based ST21CX medium. The strain DL1 showed 99% similarity to *Polyangium* sp. and 98% to *Sorangium cellulosum* strain X3T8 and was able to produce a sesquiterpene-like eremophilene. With a high potential of application, isolation and characterization of new strains of myxobacteria, especially in Vietnam, might contribute to the exploitation of novel secondary metabolites for pharmaceuticals, fragrances and agrochemicals.

Acknowledgment

The research was funded by The Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology (VAST) under the project CSK13-02.

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IN VITRO MICROPROPAGATION OF *Paramignya trimera*, A VALUABLE MEDICINAL PLANT COLLECTED FROM KHANH HOA, VIETNAM

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Received date: 20.09.2017 Accepted date: 25.10.2017

ABSTRACT

In Vietnam, root of *Paramignya trimera* has been known as a traditionally medicinal plant for treatment of various types cancers. For years, over-exploitation has led to the reduced diversity and availability of this plant resource. In nature, the multiplication rate of *Paramignya trimera* from seeds is very low. In order to increase the rate of multiplication for conservation purpose, in this study, *in vitro* micropropagation of *Paramignya trimera* was carried out. The results showed that woody plant medium (WPM) containing 20 g/l sucrose and 7.0 g/l agar was most suitable for *in vitro* propagation of *Paramignya trimera*. WPM supplemented with 3.0 mg/l BA, 0.2 mg/l TDZ was optimal medium for shoot formation while WPM supplemented with 1.5 mg/l IBA was suitable for the root induction.

Keywords: BA, IBA, *in vitro* micropropagation, *Paramignya trimera*, TDZ, the multiple shoot induction.

Nhân nhanh *in vitro* cây xáo tam phân (*Paramignya trimera*) thu thập tại tỉnh Khánh Hòa, Việt Nam

TÓM TẮT

Ở Việt Nam, rễ cây xáo tam phân (*Paramignya trimera*) được biết là dược liệu quý được sử dụng để điều trị nhiều dạng ung thư khác nhau. Tuy nhiên, do khai thác liên tục trong thời gian dài nên số lượng cây trong tự nhiên ngày càng suy giảm đến mức hầu như không tìm thấy trong tự nhiên. Cho đến nay các công trình nghiên cứu về nhân nhanh giống cây xáo tam phân còn rất hạn chế. Đối với cây xáo tam phân, hệ số nhân giống tự nhiên từ hạt rất thấp, do đó cần tìm ra phương pháp hiệu quả hơn để nhân nhanh giống cây này là rất cần thiết để cung cấp đủ cây giống cho quy mô lớn. Trong nghiên cứu này, quy trình nhân nhanh cây xáo tam phân đã được thực hiện. Kết quả cho thấy đã xác định được môi trường nền thích hợp cho nhân nhanh *in vitro* xáo tam phân là Woody plant medium (WPM). Các đoạn thân mang chồi ngủ sau khi khử trùng được chuyển sang môi trường nền thích hợp có bổ sung BA, TDZ, IBA, α-NAA trong 8 tuần. Môi trường thích hợp để nhân nhanh chồi từ đoạn thân mang chồi ngủ là WPM có bổ sung 3,0 mg/l BA và 0,2 mg/l TDZ cho tỷ lệ mẫu phát sinh chồi đạt 94,07%, hệ số nhân chồi là 3,5 chồi/mẫu. Để cảm ứng tạo rễ, các chồi *in vitro* từ 2 - 3 cm được cắt nhỏ sau đó được chuyển vào môi trường WPM có bổ sung 1,5 mg/l IBA. Kết quả cho thấy, tỷ lệ ra rễ cao nhất đạt 92,7% và số lượng rễ đạt 2,31/chồi. Kết quả thu được cho phép nhân nhanh cây xáo tam phân trong điều kiện *in vitro*.

Từ khóa: Nhân nhanh chồi, nhân giống *in vitro*, môi trường WPM, *Paramignya trimera*, ung thư, xáo tam phân.

1. INTRODUCTION

In Vietnam, *Paramignya trimera* has been widely used as a herbal medicine to treat various types of cancers and protect liver (Nguyen *et al.*, 2015; Nguyen *et al.*, 2016). As a small wild woody plant, *Paramignya trimera* distributes in mountainous regions higher than 200 m above sea level with arid climate (Pham H.H, 2003). So far, seven species of *Paramignya* were found in Ninh Hoa district, Khanh Hoa province. Recently, analysis of the chemical

profile of the *Paramignya trimera* extract revealed that this plant possesses a variety of secondary metabolites belonging to the groups of flavonoid, saponin, alcaloid, courmarin and triterpenoids (Hoang T.L.A. *et al.*, 2017; Trinh H.D. *et al.*, 2016). Some bioactive compounds such as acridone (alkaloid) and coumarin derivates were found in root of *Paramignya trimera* (Tran T.T.Q. *et al.*, 2014; Bui T.T.L. *et al.*, 2015). The root extracts showed inhibitory effect on the development of acute hepatitis and cancer cell lines such as Hep-G2, lymphoma, MDA-MB-231 and OVCAR-8. Interestingly, the root extract of *Paramignya trimera* showed no cell toxicity in mice (Nguyen M.C. *et al.*, 2016). The fact that bioactive compounds mostly exist in the root, therefore, people used root for treatment of liver diseases. As a result, the number of this plant is significantly reduced for a long time of over-exploitation. In nature, the multiplication rate of *Paramignya trimera* from seeds is low and time-consuming (Tran V.T. *et al.*, 2017). Therefore, it is neccessary to find an efficient procedure for micropropagation of *Paramignya trimera*. The objective of this study was to establish a protocol for production of large scale planting materials of *Paramignya trimera* using *in vitro* micropropagation.

2. MATERIALS AND METHODS

2.1. Explants and sterilization

Fresh young nodal segments of about 20 cm from 2 year-old seed-raised *Paramignya trimera* collected from Ninh Van, Khanh Hoa district of Viet Nam were used as explants. The nodal segments were washed under running water and soaked in sodium hypocloride solution for 15 min. The nodal segments were subsequently rinsed with autoclaved sterile water then surface-disinfected with 70% ethanol for 30 seconds and transferred to laminar flow cabinet. Nano silver solutions (Sigma aldrich, Product # 730793, 20 nm particle size, prepared at 0.02 mg/mL in aqueous buffer containing sodium citrate as stabilizer) at different concentrations were applied for 60 min (Abdi, 2012, Nayan *et al.*, 2017). Johnson solution 2.5% (prepared from Presept™ Disinfectant Tablets 2.5g - Fisher Scientific) was used to sterilize the explants with varying duration. The surface sterilized explants were rinsed with autoclaved sterile water. The efficiency of the sterilization was evaluated by the survival and contamination rate.

2.2. Culture conditions

Three basal media, MS (Murashuge-Skoog, 1962), WPM (Lloyd and McCown, 1981) and Knudson (Knudson, 1946) were used. These basal media were supplemented with 20 g/l sucrose and 7 g/l agar and pH adjusted to 5.7.

To investigate the influence of phytohormones on the proliferation and growth of explants, the WPM containing 20 g/l sucrose and 7.0 g/l agar was supplemented with various concentrations of BA (1.0 - 5.0 mg/l), TDZ (0.1 - 0.5 mg/l) and IBA (0.1 - 1.5 mg/l). The explants were cultured for 8 weeks at room temperature (25 ± 2°C) with humidity of about 70% and periodically illuminated at 14 light :10 in darkness using cool white fluorescent lamps at 2000 lux.

For callus induction, *in vitro* shoots were excised into 0.5 cm segments then transplanted to the callus induction media. The effect IBA, TDZ and 2,4-D (2,4-dichlorophenoxyacetic acid) combinations in WPM was investigated. The experiments were kept in darkness and the data were obtained after 12 weeks. The, survival rate of explants, shoots, calluses or roots was calculated based on morphological observation and plant growth.

All experiments were repeated at least three times. In each experiment at least 3 explants were planted onto a solid medium in 250 ml glass bottles. Data were collected every week including the infection rate and the rate of shoot, callus and root induction was calculated. The properties of shoots, calluses or roots were analyzed including quantity, shape, colour and quality. The means were separated by Duncan's multiple range test.

3. RESULTS AND DISUSSION

3.1. Effects of sterilization conditions

3.1.1. Effect of nano silver solution

It has been mentioned that nano silver can inhibit the growth of bacteria and fungi but not explants (Shin H.S. *et al.*, 2004; Wang G. *et al.*, 2007). In the present study, the effect of nano silver treatment for 60 min at different concentrations was investigated. The results showed that there was a significant influence of nano silver on the survival, contamination and death rate of sterilized explants (Table 1). At nano silver concentration of 200 or 300 ppm, the survival rates were 61.63 % ± 7.13 and 60.05 % ± 7.28, respectively, significantly higher than that of the control and higher concentration (400 ppm). Therefore, in this study, the concentration of 200 ppm of nano silver was selected as a suitable sterilization condition.

Table 1. Effect of nano silver on the rate of survival, contamination and death of explants

Nano silver concentration (ppm)	Survival rate (%)	Contamination rate (%)	Death rate (%)
0 (control)	0	100	0
100	53.13 ± 6.21^{b}	43.65 ± 5.05^{a}	3.22 ± 0.42^{a}
200	61.63 ± 7.13^{a}	30.00 ± 4.86^{b}	8.37 ± 1.24^{b}
300	60.05 ± 7.28^{a}	33.28 ± 5.13^{b}	6.67 ± 1.51^{c}
400	58.53 ± 6.57^{b}	31.45 ± 5.29^{b}	10.02 ± 1.36^{d}

Table 2. Effect of Johnson solution on the disinfection of explants

Treatment duration (min)	Survival rate (%)	Contamination rate (%)	Death rate (%)
0 (control)	0	100	9.07 ± 3.25^{a}
5	13.81 ± 4.17^{a}	72.41± 7.53^{a}	13.78 ± 3.19^{b}
10	15.56 ± 3.73^{a}	67.42 ± 7.15^{a}	17.02 ± 4.34^{cd}
15	13.27 ± 4.57^{a}	60.17 ± 7.46^{b}	29.56 ± 6.09^{d}
20	11.33 ± 4.24^{a}	48.65 ± 4.15^{c}	40.02 ± 6.15^{e}
25	13.67 ± 4.65^{a}	43.30 ± 6.71^{c}	45.03 ± 6.27^{f}

Table 3. Combined effect of nano silver and Johnson solution on the disinfection of explants

Combination treatment		Survival rate (%)	Contamination rate (%)	Death rate (%)
Nano silver (ppm)	Johnson solution (min)			
200 (control)	0	61.67 ± 4.43^{c}	30.00 ± 4.62^{a}	8.33 ± 1.21^{c}
200	5	62.80 ± 6.22^{c}	26.53 ± 3.66^{b}	10.67 ± 1.52^{c}
200	10	72.46 ± 8.11^{a}	2.20 ± 0.11^{c}	25.34 ± 2.35^{b}
200	15	65.03 ± 7.65^{b}	2.56 ± 1.68^{c}	32.41 ± 2.88^{b}
200	20	47.55 ± 5.25^{d}	1.43 ± 0.24^{d}	51.02 ± 4.24^{a}

3.1.2. Effect of Johnson solution

The fungicide effect of Johnson solution (2.5%) with different time duration was investigated. The data obtained two weeks after culture showed that the time of treatment was not effective in survival rate but critical in contamination and death rate (Table 2). Longer time of treatment (20 to 25 min) significantly reduced the contamination rate ($p < 0.05$) from 72.41 % to 43.30 % but accompanied with increased death rate. There was no significant difference in survival rate with regard to treatment duration.

3.1.3. Combined effect of Johnson and nano silver solution

Based on the obtained data (Table 1), explants treated with 200 ppm nano silver

solution was subsquently subjected to Johnson solution (2.5%) for varying duration. The surface-disinfected explants were then cultured on MS medium. The results obtained after 2 weeks revealed that the combination between nano silver at 200 ppm and Johnson solution (2%) for 10 min showed the highest survival rate (72.46 , $p < 0.05$). Increase in treatment time to 20 min led to a significant reduction in survival rate (47.55 %) and an increase of the death rate (51.02 %) (Table 3).

3.2. Effect of basal media on shoot induction

The results obtained after 8 weeks of culture on MS, WPM and Knudson media are shown in Table 4. Among three media, WPM was most suitable for shoot formation (Table 4). The highest rate of shoot formation was 87.96 ± 9.63% in WPM after 4 weeks. Therefore, WPM were used as the suitable basal media for the micropropagation of *Paramignya trimera*.

3.3 Effect of phytohormone on the proliferation and growth of shoots

3.3.1. Effect of cytokinin

Effect of BA: The explants were cultured on the media supplemented with BA at different concentrations (1.0, 2.9, 3.0, 4.0 and 5.0 mg/l. The differentiation of shoots appeared after about 30 days of culture. Collected data after 8 weeks are presented in table 5.

The results showed that shoots appeared after 3 - 4 weeks of culture. In the WPM supplemented with BA from 1.0 to 2.0 mg/l, the rate of shoot formation rate varied from 37.03 to 51.85 %. The optimal concentration of BA was 3.0 mg/l in terms of shoot induction rate and the average number of shoots per each explant.

Table 4. Effect of basal media on the shoot formation

Media	Shoot induction time (week)	Shoot forming rate (%)	Shoot properties
MS	5	55.60 ± 6.71^{b}	Medium, weak
WPM	4	87.96 ± 9.63^{a}	Multiple, healthy
Knudson	5	36.70 ± 5.34^{c}	Small, thin

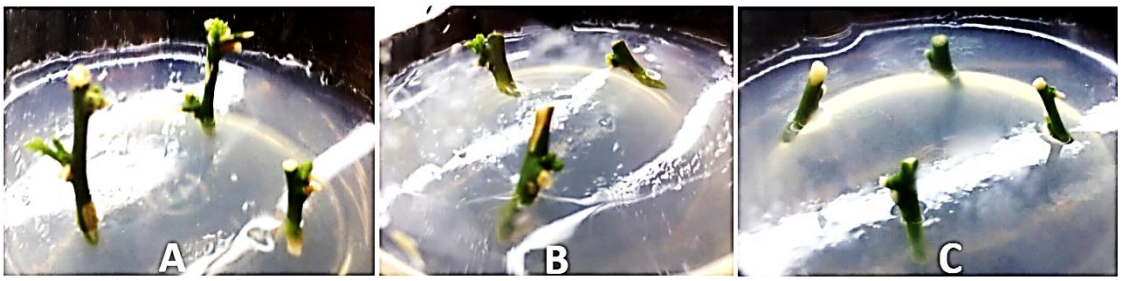

Fig. 2. *In vitro* culture of the explants on three different media WPM (A), MS (B), and Knudson (C) after 5 weeks

Table 5. Effect of BA on proliferation and growth of shoots

BA concentration (mg/l)	Shoot formation rate (%)	Number of shoots/explant	Shoot properties
0	6.01 ± 0.86^{a}	0.24 ± 0.06^{d}	Less
1.0	37.03 ± 4.25^{b}	1.81 ± 0.22^{c}	Less
2.0	51.85 ± 7.46^{c}	2.43 ± 0.23^{b}	Less, short
3.0	66.67 ± 8.15^{d}	3.17 ± 0.46^{a}	Multiple
4.0	59.26 ± 7.96^{e}	2.75 ± 0.56^{b}	Multiple
5.0	55.56 ± 7.12^{e}	2.26 ± 0.46^{b}	Multiple, weak

Note: For shoot properties, less (number of shoot < 2/explants), short (length < 0.3 cm), multiple (≥ 3/explants), weak (slow growth)

Table 6. Effect of TDZ on proliferation and growth of explants

TDZ concentration (mg/l)	Shoot formation rate (%)	Number of shoots/explant	Shoot properties
0	6.01 ± 0.96^{e}	0.24 ± 0.07^{d}	Less, small
0.1	40.74 ± 5.12^{d}	1.7 ± 0.18^{b}	Thin, bluish, succulent
0.2	59.26 ± 7.08^{a}	2.3 ± 0.26^{a}	Small, forming callus
0.3	51.85 ± 7.34^{b}	1.9 ± 0.21^{b}	Small, forming callus
0.4	48.14 ± 6.91^{c}	1.7 ± 0.19^{b}	Small, forming callus
0.5	40.74 ± 5.12^{d}	1.4 ± 0.16^{c}	Small, forming callus

Note: For shoot properties, less (number of shoot < 2/explants), small (growth slow and length < 1 cm), thin (diameter < 0,2 cm).

Effect of TDZ

TDZ enhanced the *in vitro* proliferation and growth of *Paramignya trimera* (Table 6). After 3 weeks, maximal shoot formation rate (59.26 %) and highest number of shoots per explant was observed in the WPM supplemented with TDZ at 0.2 mg/l. The results were also matching with previous studies (Guo B. *et al.,* 2011) It is also the reason why TDZ have been widely used for micropropagation and organogenesis of both annual and perennial plants (Truong T.B.P. *et al.*, 2016).

Combination effect of BA and TDZ

The explants were also cultured in WPM supplemented TDZ at 0.2 mg/l and various concentrations of BA from 1.0 to 5.0 mg/l. Collected data after 8 weeks of culture showed that the WPM supplemented with TDZ at 0.2 mg/l and BA at 3 mg/l increased the shoot proliferation and growth rates of shoots on explants (Table 7, Fig. 3). The rate of shoot formation was 92.07 ± 7.27% and the number (3.5 per explant). The combination effects of TDZ and BA were also mentioned in number of reports working with woody plants (Mehdi M. *et al.*, 2015; Reza A.G. *et al.*, 2014). In general, the concentrations of BA 3.0 mg/l and TDZ 0.2 mg/l were optimal for shoot induction in *Paramignya trimera*.

3.3.2. Combined effect of cytokinin and auxin on the shoot proliferation and growth

Combined effect of BA and α-NAA

Adding of α-NAA increased both quality and number of shoot formation (Nguyen Q.T. *et al.*, 2004; Nguyen T.L.A. *et al.*, 2007). However, in the present study, the adverse effects were observed when α-NAA was added at the concentrations from 0.5 to 1.0 mg/l and from 2.0 to 2.5 mg/l. The maximal effect on the shoot proliferation and growth were observed when α-NAA was added at concentration of 1.5 mg/l. The shoot formation rate and number of shoots per explant were 72.96 and 2.6 , respectively. Although synergic effect of cytokinin and α-NAA were often pronounced in a number of reports (Nguyen Q.T. *et al.*, 2004; Nguyen T.L.A. *et al.*, 2007), in this study the combination of BA and α-NAA significantly reduced the number of shoots per explant in comparison to the control (no α-NAA) (Table 8). Therefore, α-NAA was not used in further studies in combination with BA in further experiments.

Table 7. Combination effect of BA and TDZ on the proliferation and growth of explants

BA (mg/l)	Shoot formation rate (%)	Number of shoots/explant	Shoot properties
0	59.26 ± 7.01^{c}	2.3 ± 0.38^{d}	Small, forming callus
1	48.14 ± 5.12^{d}	2.2 ± 0.25^{d}	Thin, bluish, succulent
2	69.26 ± 7.35^{b}	2.8 ± 0.37^{c}	Small, green
3	92.07 ± 7.27^{a}	3.5 ± 0.48^{a}	Healthy, green
4	66.67 ± 7.94^{b}	3.2 ± 0.42^{b}	Healthy, green
5	55.56 ± 7.49^{c}	2.7 ± 0.28^{c}	Small, green

Note: For shoot properties, small (growth slow and length < 1 cm), thin (diameter < 0,2 cm), healthy (green)

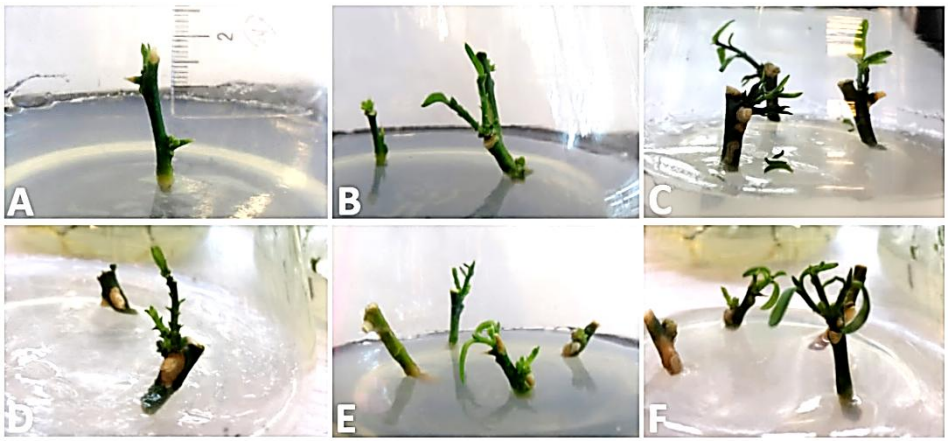

Figure 3. Combination effect of BA and TDZ on the proliferation and growth of explants

Note: Typical images of the shoot formation from explants after 8 weeks in vitro. A (control): 0 mg/l BA; B: 1.0 mg/l BA; C: 3.0 mg/l BA; D: 2.0 mg/l BA; E: 4.0 mg/l BA; F: 4.0 mg/l BA)

Table 8. Combination effect of BA and α-NAA on the proliferation and growth of explants

α-NAA (mg/l)	Shoot formation rate (%)	Number of shoots/explant	Shoot properties
0 (control)	66.67± 7.48^{b}	3.1 ± 0.38^{a}	Multiple, healthy
0.5	40.74 ± 5.03^{d}	1.5 ± 0.20^{d}	Thin, bluish, succulent
1.0	51.85 ± 6.11^{c}	1.9 ± 0.23^{c}	Medium, healthy
1.5	72.96 ± 8.18^{a}	2.6 ± 0.38^{b}	Medium, healthy
2.0	55.56 ± 6.98^{c}	2.1 ± 0.31^{c}	Small, healthy
2.5	48.14 ± 6.63^{d}	1.8 ± 0.24^{c}	Small, healthy

Note: For shoot properties, healthy (green), thin (diameter < 0,2 cm), medium (2 shoots/explant), small (shoot growth slow and length < 1 cm), multiple (≥ 3/explants)

Table 9. Combination effect of BA and IBA on the proliferation and growth of explants

IBA (mg/l)	Shoot formation rate (%)	Number of shoots per explant	Shoot properties
0 (control)	66.67 ± 7.01^{b}	3.1 ± 0.41^{b}	Multiple, healthy
0.25	44.40 ± 5.38^{d}	1.6 ± 0.12^{e}	Thin, bluish, succulent
0.5	51.85 ± 6.21^{c}	2.2 ± 0.28^{d}	Small, green
0.75	66.67 ± 7.25^{b}	2.9 ± 0.38^{c}	Healthy, green
1.0	77.78 ± 7.14^{a}	3.3 ± 0.42^{a}	Healthy, green
1.25	70.37 ± 8.12^{b}	3.1 ± 0.37^{b}	Medium, green

Note: For shoot properties, healthy (green), thin (diameter < 0,2 cm), medium (2 shoots/explant), multiple (≥ 3/explants).

Combined effect of BA and IBA

Shoot formation rate decreased from 66.67% to 44.40% when BA-containing medium was supplemented with IBA 0.25 mg/l in comparison to the control (Table 9). However, when the concentration of IBA increased, the shoot forming rate and number of shoots per explant increased and reached maximum of 77.78% at the concentration of IBA 1.0 mg/l.

3.4. Effect of auxin and cytokinin on callus formation and organogenesis

3.4.1. Effect of TDZ, IBA and 2,4-D on callus formation and organogenesis

The formation of callus from *in vitro* shoots was observed after 12 weeks of culture. The results showed that the formation of callus was observed in all auxin containing media. The highest rates of the callus formation were 70.00

%, 68.33 % and 63.33 % in the media supplemented with TDZ 1.0 mg/l, IBA 3.0 mg/l and 2,4-D 3.0 mg/l, respectively (Table 10). The presence of TDZ in the culture medium significantly stimulated the callus formation of explants. The best result obtained in media supplemented with 1.0 mg/l TDZ and callus was healthy with green color. Combination with the data obtained from previous experiments suggested that the addition of TDZ is an essential factor for the callus formation and the quality. In practice, TDZ has been also found as an effective agent for the *in vitro* callus induction (Nguyen Q.T. *et al.*, 2004).

3.4.2. Combined effect of TDZ with different concentrations of IBA and 2,4-D on the callus formation

In order to increase the number and quality of callus, a combination of TDZ (1.0 mg/l, control) with various concentrations of IBA and 2,4-D was carried out. The results were presented in Table 11. There was significant interaction between TDZ and 2,4-D on callus formation. The rate of callus formation increased from 70.01 % to 86.67 % when 3.0 mg/l 2,4-D was added. Higher concentration of 2,4-D likely reduced the rate of callus formation. When IBA was added, the rate was not significantly changed (80.47 % to 93.33 %). However, the calluses regenerated from the medium containing 1.0 mg/l TDZ and 3.0 mg/l 2.4-D had better quality with green color. In contrast, in the media supplemented with other concentrations of IBA, calluses were weak with greenish colour (Table 11, Fig. 4).

3.5. Effect of IBA and α-NAA on root induction from *in vitro* shoots

Investigation of root induction using WPM containing various concentrations of α-NAA (0.5 to 2.0 mg/l) and IBA (0.5 to 2.5 mg/l) was carried out. The results showed that the effect of IBA on root formation was significantly higher than that of α-NAA (Table 12). However, effect of auxin on root induction was unstable and required much time (8 weeks). In addition, after 8 weeks, the number of roots per shoot and root length (data not shown) were remarkably different among treatments (Table 12 and Figure 5). After 12 weeks, induced roots elongated and the number of roots per shoot reached 2.31. The highest rate of root formation was 92.71 % on the WPM supplemented with 1.5 mg/l IBA (Table 12).

Table 10. Effect of TDZ, IBA and 2.4-D on callus formation and organogenesis

Auxin	Concentration (mg/l)	Survival rate of planted segments (%)	Callus formation rate (%)	Callus properties
Control	0	91.05 ± 9.86^{b}	-	-
TDZ	0.5	96.67 ± 11.74^{a}	61.67 ± 7.05^{c}	Healthy, green
	1.0	91.67 ± 10.09^{b}	70.00± 8.16^{a}	
	1.5	88.33 ± 9.23^{b}	60.67 ± 7.96^{c}	
	2.0	85.12 ± 8.97^{c}	63.33 ± 7.65^{c}	
IBA	1.0	92.46 ± 10.74^{b}	56.67 ± 6.02^{b}	Weak, white
	2.0	90.15 ± 9.76^{b}	61.67± 7.41^{c}	
	3.0	86.67 ± 9.54^{c}	68.33 ± 7.95^{b}	
	4.0	85.17 ± 9.86^{c}	66.67 ± 7.05^{b}	
2,4-D	1.0	91.67 ± 10.43^{b}	58.33 ± 6.66^{d}	Weak, yellow
	2.0	88.33 ± 9.01^{c}	61.67 ± 8.11^{c}	
	3.0	83.33 ± 10.35^{c}	63.33 ± 7.54^{c}	
	4.0	80.02 ± 9.78^{c}	60.04 ± 7.05^{c}	

Note: For callus properties, healthy (green and grow fast), weak (callus grow slow and greenish or white or yellow. (-): No callus formation

Table 11. Effect of the combination between TDZ, IBA and 2,4-D on callus formation and organogenesis

Auxin hormones	Concentration (mg/l)	Survival rate (%)	Callus formation rate (%)	Callus properties
1.0 mg/l TDZ (control)	1.0	91.67 ± 10.09^{b}	70.01 ± 8.45^{b}	-
2,4-D	2.0	95.01 ± 9.78^{a}	63.33 ± 8.14^{c}	Healthy
	3.0	91.67 ± 8.16^{b}	86.67 ± 9.07^{a}	
	4.0	90.23 ± 8.25^{b}	81.67 ± 9.15^{a}	
IBA	1.0	93.33 ± 8.04^{a}	66.67 ± 7.08^{c}	Medium
	2.0	86.67 ± 7.95^{b}	70.28 ± 8.76^{b}	
	3.0	85.64 ± 8.11^{b}	73.33 ± 8.57^{b}	
	4.0	80.47 ± 7.13^{c}	68.33 ± 7.01^{b}	

Note: For callus properties, healthy (green), medium (greenish). (-): No callus formation

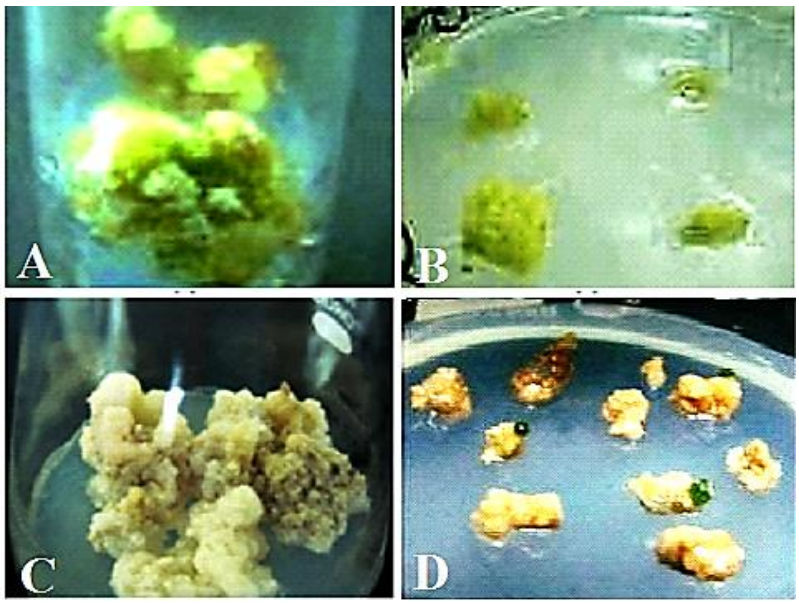

Fig 4. The formation of callus from *in vitro* shoots after 12 weeks

Note: The morphology of calluses formed on the media supplemented with 1.0 mg/l TDZ and 3.0 mg/l 2.4 D (A, B); 1.0 mg/l TDZ and 3.0 mg/l IBA (C, D).

Table 12. Effect of IBA and α-NAA on the induction and growth of roots from *in vitro* shoot

Auxin hormones (mg/l)		Root induction rate (%)	Number of roots/shoot	Root properties
WPM (control)	0	0	0^{a}	-
α-NAA	0.5	11.62 ± 1.24^{c}	0.21 ± 0.25^{a}	White
	1.0	22.11 ± 2.14^{b}	1.02 ± 0.11^{b}	White, weak
	1.5	42.60 ± 5.03^{a}	1.16 ± 0.14^{b}	White
	2.0	40.12 ± 4.96^{a}	1.11 ± 0.14^{b}	White, weak
IBA	0.5	21.23 ± 3.02^{d}	0.76 ± 0.08^{c}	White
	1.0	52.18 ± 6.56^{c}	1.12 ± 0.12^{b}	White, healthy
	1.5	92.71 ± 7.67^{a}	2.31 ± 0.24^{a}	Medium, healthy
	2.0	88.62 ± 9.04^{a}	1.96 ± 0.14^{a}	Medium, healthy
	2.5	71.29 ± 8.14^{b}	1.21 ± 0.15^{b}	White

Note: For root properties, weak (length < 1 cm and grow slow) medium (length < 2), healthy (≥ 2.5 cm). (-): No root formation

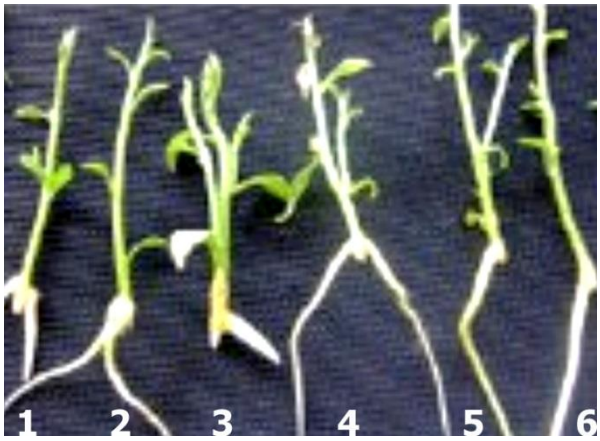

Figure 5. Effect of IBA on the induction and growth of roots from *in vitro* shoots after 12 weeks

Note: Different concentrations of IBA in WPM were used: 1 (0 mg/l); 2 (0.5 mg/l); 3 (1.0 mg/l); 4 (1.5 mg/l); 5 (2.0 mg/l) and 6 (2.5 mg/l).

4. CONCLUSIONS

Nano silver solution 200 ppm was appropriate and effective for the sterilization of explants from *Paramignya trimera.* When subsequently treated with Johnson solution 2.5% for 10 minutes, the explants were sterilized more effectively in terms of survival rate of explants. For *in vitro* micropropagation, the most suitable basal medium was WPM and WPM supplemented with 3.0 mg/l BA and 0.2 mg/l TDZ was suitable for shoot induction. Calli in WPM supplemented with 3.0 mg/l 2.4 D and 1.0 mg/l TDZ were healthy and had green colour. The optimal medium for root induction was WPM supplemented with 1.5 mg/l IBA.

Acknowlegement: This project was funded by AI Programme with Vietnam National University of Agriculture.

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Vietnam J. Agri. Sci. 2017, Vol. 15, No. 9: 1266 - 1276 Tạp chí Khoa học Nông nghiệp Việt Nam 2017, 15(9): 1266 - 1276
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HOUSEHOLD FOOD SECURITY AND LIVELIHOOD STRATEGIES IN PROTECTED AREAS: A CASE STUDY IN TAY YEN TU NATURE RESERVE, SON DONG DISTRICT, BAC GIANG PROVINCE

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Received date: 27.02.2017 Accepted date: 05.05.2017

ABSTRACT

The main objective of this study was to quantify food security at the household level in Tay Yen Tu Nature Reserve, Son Dong district, Bac Giang province. The Household Food Insecurity Access Scale (HFIAS) was used to obtain food insecurity statuses. Households in Tay Yen Tu Nature Reserve were classified into four categories including food security, mild food insecurity, moderate food insecurity, and severe food insecurity with prevalences of 25.28%, 20.8%, 32.5%, and 20.8%, respectively. The relationship between livelihood strategies and household food security (HFS) revealed that diversifying products and sales tended to improve the food security status. Furthermore, using Principal Component Analysis (PCA) and Cluster Analysis (CA), the research found that the livelihood strategies of the food security category were based on agricultural and natural forest activities, while those of the food insecurity categories related to commercial forest, off-farm, and other activities. Finally, this research suggested that policy makers should focus on promoting agricultural models in paddy rice, maize, peanut, and livestock production; provide support for poor households cultivating plants that consume less water such as soybean, maize, and peanut; shift traditional cultivation from “cereal-livestock mix” to a model of cash income diversification; and invest in and implement intensive horticultural production and infrastructure development including transportation development, irrigation systems, electricity, and market development.

Keywords: Food security, livelihood strategies, protected area

An ninh lương thực cấp hộ và chiến lược sinh kế ở các khu bảo tồn: trường hợp nghiên cứu tại khu bảo tồn thiên nhiên Tây Yên Tử, huyện Sơn Động, tỉnh Bắc Giang

TÓM TẮT

Mục tiêu chính của nghiên cứu là đo lường an ninh lương thực cấp hộ ở khu bảo tồn thiên nhiên Tây Yên Tử, huyện Sơn Động, tỉnh Bắc Giang dựa trên thang đo tiếp cận mất an ninh lương thực cấp hộ (HFIAS).Các hộ nghèo điều tra ở Khu bảo tồn được phân loại thành 4 cấp độ bao gồm: an ninh lương thực, không đảm bảo an ninh lương thực ở mức độ nhẹ, mức độ trung bình và mức độ trầm trọng, với tỉ lệ lần lượt là 25,28%, 20,80%, 32,50% và 20,8%. Xem xét mối quan hệ giữa an ninh lương thực cấp hộ và chiến lược sinh kế, nghiên cứu phát hiện ra rằng việc đa dạng hóa sản phẩm và nơi bán giúp cho hộ cải thiện tình trạng an ninh lương thực. Bên cạnh đó, sử dụng phương pháp phân tích thành phần chính (PCA) và phân tích cụm (CA), nghiên cứu chỉ ra rằng chiến lược sinh kế của các hộ an ninhlương thực thường sử dụng là dựa vào các hoạt động nông nghiệp và rừng tự nhiên. Trong khi đó, các hộ mất an ninh lương thực thì dựa vào thu nhập từ rừng sản xuất và thu nhập phi nông nghiệp. Từ đó, nghiên cứu đề xuất khuyến nghị chính sách như: hỗ trợ phát triển các mô hình trình diễn trong nông nghiệp; hỗ trợ cho hộ nghèo canh tác cây trồng sử dụng ít nước như ngô và cây trồng họ đậu; chuyển đổi sản xuất từ kết hợp cây ngũ cốc - chăn nuôi sang mô hình đa dạng hóa thu nhập; cải tạo vườn tạp và phát triển hạ tầng bao gồm: giao thông, thủy lợi, điện và phát triển thị trường.

Từ khóa: An ninh lương thực, chiến lược sinh kế, khu bảo tồn.

1. INTRODUCTION

Food security has been a global issue attracting much attention in many countries around the world. Food security exists when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life (FAO, 1996). According to FAO, Vietnam has been food-secure at a national level since 1990 but in fact, 9.9 million people still remained undernourished in 2015 in Vietnam, occupying 11 percent of Vietnam's population. Thus, food security at an individual and household level continues to be a problem in Vietnam, especially in the remote areas of this country (WorldBank, 2015).

Tay Yen Tu Nature Reserve is located in Northeast Vietnam. It was established by the Decision No. 117/QD-UB of Bac Giang Provincial People's Committee on July 22, 2002. Tay Yen Tu Nature Reserve is considered to be a high biodiversity area in Northeast Vietnam. About 45% of its population living in or around the nature reserve was poor and highly dependent on forests (Forest Protection Department of Bac Giang Province, 2016). Thus, starvation and poverty have led to the conversion of forest to agricultural land, soil erosion, illegal logging, and environment degradation. Through the years, these phenomenon have led to the decreases in the quantity and quality of the ecosystem. Degradation of the environment, biodiversity loss, poverty, and food insecurity have become the big issues that are challenging policy decision-making. With all the reasons mentioned above, finding out the relationship between household food security and livelihood strategies is expected to contribute to solving the trade-off between conservation and food security in protected areas in Vietnam.

2. METHODOLOGIES

2.1. Study Site Selection

Tay Yen Tu Nature Reserve is located in four communes: Thanh Son, Thanh Luan, Tuan Mau, and An Lac of Son Dong district and Luc Son commune of Luc Nam district, Bac Giang province. The nature reserve is included on a list of special-use forests of Vietnam. This nature reserve consists of two sections: Tay Yen Tu and Khe Ro, with a total forest area of 13,022 ha, comprised of a 6,022 ha core zone and a 7,000 ha ecological rehabilitation zone.

Tuan Mau, Thanh Son, and An Lac were the 3 sampled communes. These communes were selected as the research sites because of the following reasons: i) these communes were located in both inside and outside the nature reserve, ii) the three communes showed the highest poverty proportion in the area at 34.65%, 44.62%, and 53.56%, respectively, in 2015 (Commune People's Commitee of Son Dong District, 2016); and iii) the livelihoods of the households living in those communes strongly depended on natural resources of the nature reserve. In terms of section, the nature reserve had two sections: Thanh Luc Son and Khe Ro. The proportion of poverty in the Thanh Luc Son section was lower than that of the Khe Ro section.

2.2. Data Collection

Both primary and secondary data were used in the study. The secondary data was collected from government offices at the commune, district, and national levels. Copying and taking photographs helped to collect most documents concerning food security in protected areas. The Participatory Rural Appraisal (PRA) tools and a household survey were used to collect the primary data. As for sampling design, the targeted sample was poor households in The Tay Yen Tu Nature Reserve. The population was the 1,007 households below the poverty line. According to the sample size determination by Krejcie & Morgan (1970), at a 5% level of significance and a t-value of 1.96 derived from a population size of 1000 of continuous data (cited by Bartlett *et al.*, 2001), the sample size households in each commune of this study is given in Table 1. Simple random sampling was used to select the households in the three communes.

Table 1. Sample size

The Nature Reserve Section	Commune	Total households	Households below the poverty line	Sample Size (Households)
Thanh Luc Son	Thanh Son	520	284	25
	Tuan Mau	785	272	35
Khe Ro	An Lac	842	451	60
Total		2,147	1,107	120

Source: Author's own elaboration, 2015

2.3. Data Analysis

2.3.1. Measuring Household Food Security

The household food insecurity access scale (HFIAS), a nine-item food insecurity scale that was developed by USAID, was employed to assess household food security status in this study. The questions follow a progression, starting with anxiety about food supply, followed by questions concerning the quality of food, then questions on the quantity of food consumed, and then asking about the number of days households experienced hunger in the hunger period (60 days) during 2015[1](Deitchler *et al.*, 2010). The HFIAS indicator categorizes respondents into four levels of household food security: secure, and mildly, moderately, and severely insecure. Food security was identified if the household head said "no" to the all questions or said yes but rarely to question 1. Mild food insecurity was mentioned if the respondent said "yes, sometimes" to question 1, was not able to meet the kind of food he/she preferred, ate a limited variety of food 1 to 2 times, or just only ate some food with a frequency of rarely. Moderate food insecurity was calculated when a household said "yes, sometimes or often" to questions 3 and 4, and answered questions 5 and 6 with less than 10 times in the hunger period. Severe food insecurity occurred when a household affirmed they often have to eat a smaller meal or eat fewer meals in a day. These categories are shown in detail in Table 2.2.3.2. Identifying the relationship between food security and livelihood strategies

Principal Component Analysis (PCA)

According to Jolliffe (2014), PCA is a variable reduction procedure that transforms a number of correlated variables into a smaller number of uncorrelated variables called principal components. In this study, PCA was conducted with six variables of income sources including crop, livestock, natural forest, commercial forest, off-farm, and other income sources. The result of the PCA revealed the components representing the features of livelihood strategies based on income sources.

Cluster Analysis (CA)

CA is a tool used to identify homogenous groups of cases, such as observations and respondents, in which the same response will be allocated in a group that has the same particular features. CA is usually used based on PCA results. In this study, CA with the component scores (from PCA) was used to classify subjects into groups.

3. RESULTS AND DISCUSSIONS

3.1. Descriptive Statistics of Characteristics of Poor Households

The household's socioeconomic characteristics are shown in Table 3. Out of 120 sampled households, there were 53% households living inside in the nature reserve. These households were in four villages including Dong Thong, Dong Ri, Tan Lap, and Na Trang. Other households, accounting for 47%, belonged to Neo, Na O, and Thac villages. Male household heads accounted for 82.5% of the respondents.

[1]The hunger period was determined based on results of group discussions with leaders of villages and households to identify what time of the year the community/household is not able to get enough food for meals.

Table 2. Categories of food insecurity

Ques. No.	Content	Frequency		
		Rarely (1-2 times)	Sometimes (3-10 times)	Often (>= 10 times)
1	How often did you worry that your household would not have enough food?			
2	How often did you or any household member not able to eat the *kinds of foods you preferred*[2] because of a *lack of resources*[3]?			
3	How often did you or any household member have to eat *a limited variety of foods*[4] due to a lack of resources?			
4	How often did you or any household member have to eat some foods *(sweet potato, rice porridge, cassava root, broken rice)* because of a lack of money to obtain other types of food?			
5	How often did you or any household member have to eat a smaller meal (major eating occasions) than you felt you needed because there was not enough food?			
6	How often did you or any other household member have to eat fewer meals in a day because there was not enough food?			
7	How often did your household food stores ever completely empty and there was no way of getting more?			
8	How often did you or any household member go to sleep at night hungry because there was not enough food?			
9	How often did you or any household member go whole day and night without eating anything because of not enough food?			

Note: : Food secure; : Mildly food insecure; : Moderately food insecure; : Severely food insecure

Source: Adapted from Deitchler et al., 2010

Table 3. Household distribution by socioeconomic characteristics

Household Characteristics		Frequency	Percentage
Zonation	Outside	56	46.67
	Inside	64	53.33
Gender of household head	Female	21	17.50
	Male	99	82.50
Ethnicity	Minorities people	84	70.00
	Kinh people	36	30.00
Housing type	Semi-firm	75	62.50
	Cottage	41	34.17
	Permanent	4	3.33
Number of main assets	1-3 assets	62	51.67
	More than 4 assets	58	48.33

Source: Author's survey, 2015

[2] Mean number of foods that food secure people eat that food insecure people cannot afford to eat.

[3] Mean number of people not having money or the ability to grow or trade for food.

[4] Mean of an undesired monotonous diet

Table 4. Descriptive statistics of socioeconomic characteristics

Indicator	Unit	Mean	SD	Maximum	Minimum
Age of household head	Year	43.12	13.39	83	23
Education of head household	Year	4.67	2.93	12	0
Household size	Person	3.93	1.20	7	1
Dependent ratio	%	60.12	49.11	200	0
Distance to market	km	6.25	1.67	9.3	2.3
Cropping intensity	Times	1.66	.33	2.50	1.00
Forest land size	ha	1.89	1.72	6.0	360
Cropland size	m^2/household	2,620.76	1,554.80	7,920.0	0.00
Total income	(mil. VND)	19.19	13.63	67.78	1.11

Source: Author's survey, 2015

In terms of ethnicity, five ethnic groups are living together in the nature reserve involving Kinh, Dao, Tay, Nung, and San Chi. The Kinh group is the majority ethnic group and the others are all minority ethnic groups. The minority ethinic households occupied about 70% together, while the Kinh households held 30% because the Kinh people are immigrants to this area. The Kinh people entered into this area within the four last decades, following the policies of building new economic zones in mountainous areas. Three housing types were popular in this area: semi-firm, cottages, and permanent houses with percentages of 62.5%, 34.0% and 3.5% of sampled households, respectively.

The age of household heads ranged from 23 to 83 years, 43.12 years on average, indicating that age is advantagous for economic development (Table 4). Meanwhile, schooling years of the household heads was 4.67 years on average. Most household heads stopped at primary school, and more importantly, there were 17 illeterrate household heads. Household size on average was 3.93 persons. More over, the high ratio of dependents in the households accounted for the burden on the households' labors and directly influenced food security.

The descriptive statistics of sample size showed that the mean distance to market was 6.25 km (distance from house to center market of the region) meaning that a lot of communities are living far from the commune's center. The cropping intensity was an important indicator to evaluate rotation avalability of agricultural land. This indicator depend on irragation avalability as well as crop rotation of households. In the sample size, the average cropping intensity reached 1.6 times. This could be explained by the limitation of irrigation systems, leading to housholds only able to cultivate during one season.

In terms of land size, cropland was fragmented and there currently is not a comprehensive strategy for land consolidation in local areas. According to the leader of An Lac commune, each household owned 6-7 pieces of land. The land fragmentation issue was popular in all communes, leading to many challengers in terms of machine application as well as production commercialization. The average crop land size per capita was very low (2,620 m^2/household). The backward irrigation system also leads to low productivity.

In general, the total annual income of poor households in the nature reserve reached 19.19 milllion VND per household. This number was slightly lower than the poverty standard of the whole nation (less than 0.4 million VND/capita/month).

3.2. Household Food Insecurity Status

As shown in Figure 1, the affirmative responses of household experiences were

grouped into four categories of food security. The results illustrate that the prevalence of food security, mild insecurity, moderate insecurity, and severe insecurity were 25.8%, 20.8%, 32.5% and 20.8%, respectively. The data also shows that the proportion of food insecurity of households was very high (approximately 75%) compared to the food security scale.

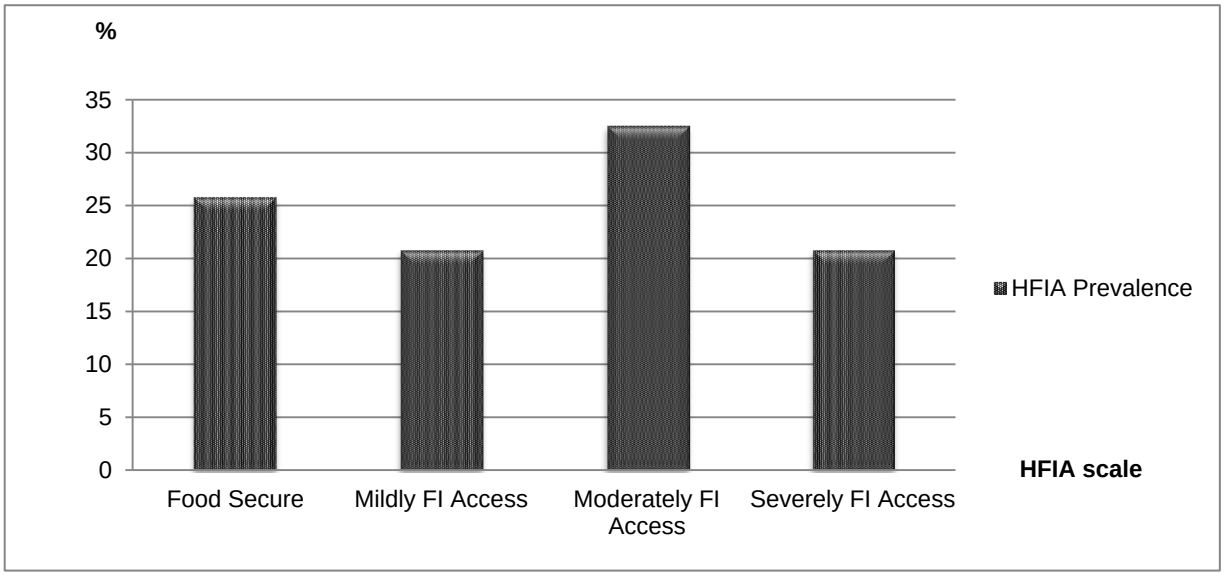

Figure 1. Household food security status

Note: FI: Food Insecurity
Source: Author's survey, 2015

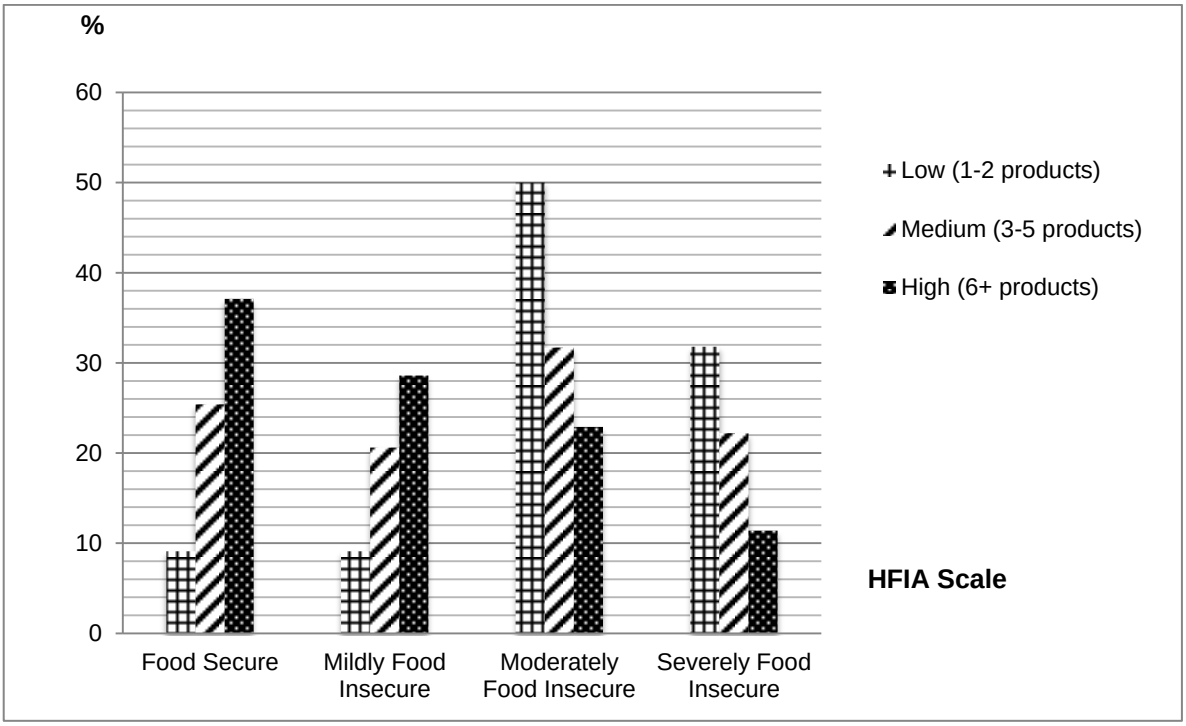

Figure 2. Production Diversification and Food Security

Source: Author's survey, 2015

3.3. Livelihood Strategies and Food Security

3.3.1. Production Diversification Strategies and Food Security

The indicators of diversity of agricultural production were derived from the number of agricultural products being produced on-farm (Barahona *et al.*, 2011). The households producing 1 to 2 product types were classified as *low production diversification*, those producing 3 to 5 product types were classified as *medium production diversification*, and the households producing 6 or more product types were considered as *high production diversification*.

In the study area, there were a total of eight major productive activities that contributed to total income of households, including paddy rice, maize, peanut, cassava, acacia, pig, poultry, and buffalo production. Of 120 households, only 8.3% of those had more than 7 product types. Most households produced from 2 to 6 product types.

After the relationship between the diversification in agricultural production activities and the level of food security was explored, we found a strong association (X^2 = 12.49, d_f = 6, $p<0.05$). The more that households diversified their products, the less food insecure those households had. The reverse was true for the less product-diversified households (Figure 2). A higher percentage of food secure households had highly diversified agricultural strategies. One-third of extremely severely food insecure households were involved in very few agriculture-related activities. Figure 2 also suggests that there may be some causes for concern over the fact that more than one-half of the households that had moderate food insecurity, fall into the 'low' diversification category.

3.3.2. Sale Diversification Strategies and Food Security

The indicators of sales activities were also derived from the number of products sold by the households. The households selling no products were classified as *subsistence,* one to two products as *low market orientation*, and three or more as *high market orientation*.

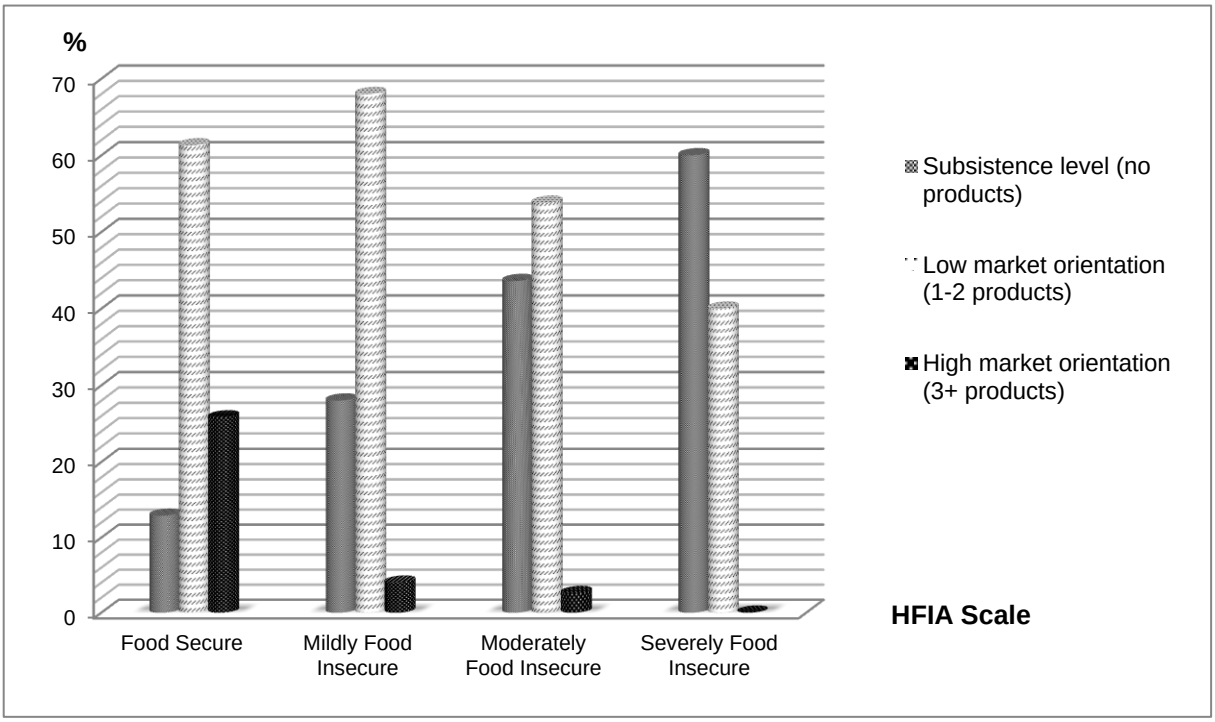

Figure 3. Sales Diversification/Market Orientations and Food Security

Source: Author's survey, 2015

Indeed, we confirmed there was a strong association between the market orientation of a household and the level of hunger reported (X^2 = 27.24, d_f = 6, p<0.001). The households selling a greater range of products reported a greater food security level; and the reverse was true for households selling fewer products. Out of 25 households that reported severe food insecurity, 60% reported that they sold no products (see Figure 3). A further 40% only sold one or two different product types.

3.3.3. Income Source-Based Strategy and Food Security

The study tried to evaluate livelihood strategies through the income sources of household, of which six income sources were determined including: *i) Crop income* was calculated from the total gross crop income minus the total cost of inputs of all crops. Total gross income was identified by value of all crop products over the year, based on local market prices at the survey time. Total cost of crop inputs was the sum of seeds, fertilizers, pesticides, plough services, etc.; *ii) Livestock income* was the total value of gross income of livestock after deducting total costs. Livestock income was formed from three main sources: livestock sales, livestock for subsistence, and livestock services (ploughing). Annual cost of livestock consisted of feeds, breeding, and veterinary services. The value of gross income and costs was also based on local market at the survey time; *iii) Natural forest income* was determined from the value of natural forest products such as fuel wood charcoal, construction wood for houses, and non-timber forest products (honey, medicinal plants, resin, tree roots, mushrooms, etc.); *iv) Commercial forest income* was from forest harvesting that households earned by casual hired labor; *v) Off-farm income* was from wages that households got through masonry, carpentry, brick making, and other rural services; and *vi) Other income* was from remittances, pensions, and subsides from poverty reduction policies for poor households.

The first step, Principal Component Analysis (PCA), was used to explore the main components from the six income sources. The results from PCA with the Varimax rotational method revealed three components with Eigen values greater than 1 (1.38, 1.24, and 1.11) which could explain 62.59% of the variance. It can be easily seen that the difference among components loading allocation in each component (Table 5 and Table 6).

Table 5. KMO and Bartlett's Test

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		0.478
Bartlett's Test of Sphericity	Approx. Chi-Square	27.582
	df	15
	Significant	0.024

Source: Author's survey, 2015

Table 6. Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	1.389	23.157	23.157	1.389	23.157	23.157	1.357	22.619	22.619
2	1.247	20.787	43.944	1.247	20.787	43.944	1.200	20.000	42.619
3	1.119	18.647	62.591	1.119	18.647	62.591	1.198	19.972	62.591
4	0.847	14.113	76.704						
5	0.789	13.144	89.849						
6	0.609	10.151	100.000						

Note: Extraction Method: Principal Component Analysis.
Source: Author's survey, 2015

Table 7. Rotated Component Matrix[a]

Income source	The most important component loading (In bold)		
	1	2	3
Crop income	0.62	-0.08	0.28
Livestock income	0.79	-0.05	-0.10
Natural forest income	-0.02	-0.29	0.76
Commercial forestry income	-0.10	-0.45	-0.69
Off-farm income	0.51	0.61	-0.22
Others income	-0.23	0.73	0.02

Note: Extraction Method: Principal Component Analysis; Rotation Method: Varimax with Kaiser Normalization; a. Rotation converged in 5 iterations.
Source: Author's survey, 2015

Table 8. Distribution of income sources among clusters

Income source	Mean income per capita (Mil.VND)		F (T-test)	Sig.
	Cluster 1 (N = 49)	Cluster 2 (N = 67)		
Crop	0.2	0.7	11.7	0.001
Livestock	0.3	0.8	14.3	0.000
Natural forest	0.1	1.4	37.7	0.000
Commercial	1.0	0.1	40.9	0.000
Off-farm	2.4	0.7	22.9	0.000
Others	1.1	0.6	11.7	0.001

Source: Author's survey, 2015

The components loading from PCA were rotated using the rotated component matrix. The result reveals three important components (Table 7). The first component had a positive significant loading related to crop income and livestock income. The second component had a positive loading in terms of off-farm income and others income. The last component showed a positive loading on natural forest income and negative loading on commercial forest income. Consequently, component 1 could be described as the "*Agricultural dependency*"; component 2 named "*Off-farm and others source dependency*" and component 3 is related to "*Forest dependency*".

The second step, the hierarchical cluster analysis using Ward's method with inputs as component scores by PCA results was used. The criteria to decide the number of clusters was based on: (i) scree plot that was made from plotting the coefficients and number of clusters, (ii) dendrogram data (Mooi & Sarstedt, 2011), and (iii) the distribution among cluster performances (Patrício *et al.*, 2013)

Cluster analysis revealed three distinct clusters. However, one cluster only had four respondents. Thus, it was excluded from the analysis. Hence, crosstabulation was conducted among the income variables and household food security statuses, and revealed two livelihood strategy groups as indicated in Table 8.

Cluster 1 represented households that had a low income from agricultural sources (crop and livestock) and natural forest sources but they earned a high commercial forest income, off-farm income, and other income. Thus, we could conclude that cluster 1 reflected the household group with commercial forest - based income, off-farm income, and other income strategies.

Table 9. Relationship between Food Security and Income Source-Based Strategy

HFIA Scale	Strategy based on commercial forest, off-farm and other activities (N = 49)		Strategies based on Agriculture, natural forest activities (N = 67)	
	Count	%	Count	%
Food Secure	7	14.3	22	32.8
Mildly Food Insecure	10	20.4	15	22.4
Moderately Food Insecure	21	42.9	16	23.9
Severely Food Insecure	11	22.4	14	20.9

*Note: * Pearson Chi-square test (Significant at p-value <0.05)*
Source: Author's survey, 2015

Conversely, cluster 2 consisted of households having a higher income from agriculture and natural forest sources. They earned less income from commercial forests, off-farm activities, and other income activities, compared to cluster 1. Hence, households in cluster 2 had agriculture-based income and natural forest-based income strategies.

The response of households regarding the household food insecurity scale (HFIA scale) to provide the relationship between livelihood strategies and food security is indicated in Table 9. The higher the proportion of households who were living on commercial forests, off-farm activities, and others activities, the more the households faced food insecurity. Particularly, more than 84% households depended on these livelihood strategies were facing food insecurity at a mild or more serious level. On the other hand, households with agricultural and natural forest strategies tended to have a higher food security status. Only one-fifth of households fell into the severe food insecurity category while approximately one-third of households ensured food security over time.

4. CONCLUSIONS

The issue of household food security and its relationship with livelihood strategies are the main concerns in Tay Yen Tu Nature Reserve. Based on the household food insecurity access scale, four categories were clarified as: food security, mildly food insecure, moderately food insecure, and severely food insecure with the prevalence of, respectively, 25.28%, 20.8%, 32.5%, and 20.8%. The results reveal that there are strong relationships between livelihood strategies and household food security. Poor households selected production diversification as the adaptive strategy as well as risk reduction strategy to ensure food security. Based on the diversification of products, households could reduce food shortage situations and mono-food intake. In addition, the research also illustrates that sale diversification contributed to ensuring food security. High proportions of respondents in the severely food insecure category depended on subsistence production while the food security group produced both subsistence and market products. Moreover, agricultural income and natural forest income were the main income sources of the food security group, while off-farm, commercial forest, and other income activities were the main income sources of the food insecurity group. The findings demonstrate that commercial forest as well as off-farm income are only temporary solutions to solve food shortage immediately. Unstable employment of off-farm jobs and low income of forest plantation are causes leading to low total income that directly affects food security. It is not in doubt that agriculture and natural forest resource still play the most important roles for food security.

Consequently, the research suggested that policy makers should build and promote the demonstration models in paddy rice, maize,

peanut, and livestock production. The efficiency of the models helps poor households be more confident to replicate. Additionally, the local government should support households cultivating plants that consume less water such as soybean, maize, and peanut. These plants not only help to diversify products but also to improve land quality. Moreover, agricultural policy should shift from a traditional cultivation of "cereal-livestock mix" to the model of cash income diversification. For example, in Son Dong district, off-farm businesses, honey production, poultry, and horticulture should be promoted widely to enhance total income as well as ensure environmental objectives. On the other hand, intensive horticultural production is not the only possible way to solve food insecurity in long-term. Some agro- forestry that should be promoted include a combination of litchi and honey production or livestock mixed production forest. Finally, policies on infrastructure including transportation development, irrigation systems, electricity, and market development should be invested and implemented.

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ECONOMIC PERFORMANCE OF COFFEE AND PEPPER INTERCROPPING IN QUANG HIEP COMMUNE, CUMGAR DISTRICT, DAK LAK PROVINCE

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Received date: 17.07.2017 Accepted date: 20.08.2017

ABSTRACT

Coffee and pepper are key crops generating income and employment for farmers in the Central Highlands. The intercropping of the two crops has been practiced recently by farmers and this has initially shown to be a good production system. The study was designed to evaluate the economic performance of coffee and pepper intercropping in Quang Hiep commune, CuMgar district, Dak Lak province, and propose key recommendations to improve the economic performance of the system. In-depth interviews with 50 farm households were conducted, and evaluated using descriptive and comparative statistics. The results show that the average area of intercropping is estimated at about 1.5 ha/ farm household. On average, a hectare of intercropping produces 2.76 tons of coffee and 1.51 tons of pepper, generating an income of about 203 million VND/ha for households during the crop season 2014 - 2015. The system proved to be more economically efficient than the mono-system. Economic performance of the intercropping system was found to be different between households and depended on the production scale, irrigation status, seed quality, gender, ethnicity, and farmers' technical know-how. The production system now faces other obstacles, such as crop diseases and pests, changing weather, input and output market, and market information. Key recommendations to improve economic performance of coffee-pepper intercropping in Quang Hiep commune are proposed accordingly.

Keywords: Coffee, economic performance, intercropping, pepper, Quang Hiep.

Hiệu quả kinh tế mô hình trồng xen hồ tiêu và cà phê tại xã Quảng Hiệp, CưMgar, Đăk Lăk

TÓM TẮT

Cà phê và hồ tiêu là hai loại cây trồng chủ lực, tạo thu nhập và việc làm cho nông dân vùng Tây Nguyên. Xen canh cà phê với hồ tiêu là phương pháp đã được áp dụng gần đây và cũng đã chứng tỏ một mô hình sản xuất tốt. Nghiên cứu này nhằm đánh giá hiệu quả kinh tế của mô hình trồng xen canh cây cà phê và hồ tiêu tại xã Quảng Hiệp, huyện CuMgar, tỉnh Đắc Lắk và đề xuất một số giải pháp nhằm nâng cao hiệu quả kinh tế của mô hình này trong thời gian tới. Nghiên cứu này được thực hiện qua điều tra 50 hộ nông dân và phỏng vấn sâu với một số tác nhân. Các phương pháp chủ yếu sử dụng là thống kê mô tả, thống kê so sánh. Kết quả cho thấy quy mô sản xuất trung bình mỗi hộ khoảng 1,5 ha. Mỗi hec-ta trồng xen canh cho sản lượng 2,76 tấn cà phê và 1,51 tấn hồ tiêu, mang lại thu nhập khoảng 203 triệu đồng/ha cho hộ nông dân trong niên vụ 2014 - 2015. Mô hình trồng xen cà phê và hô tiêu cũng chứng tỏ là có hiệu quả kinh tế hơn là các mô hình trồng độc canh. Hiệu quả kinh tế của mô hình trồng xen canh cà phê và hồ tiêu khác nhau giữa các loại hộ và phụ thuộc vào quy mô sản xuất, điều kiện nước tưới, chất lượng hạt giống, giới và dân tộc của chủ hộ, cũng như hiểu biết kỹ thuật của nông dân. Mô hình trồng xen canh cũng chịu ảnh hưởng của các yếu tố bên ngoài khác như dịch bệnh cây trồng, thay đổi thời tiết, biến động thị trường đầu vào, đầu ra, thông tin thị trường. Trên cơ sở đó, các giải pháp đã được đề xuất nhằm nâng cao hiệu quả kinh tế của mô hình xen canh cà phê và hồ tiêu.

Từ khóa: Cà phê, hồ tiêu, hiệu quả kinh tế, Quảng Hiệp, trồng xen.

1. INTRODUCTION

Perennial industrial crop production has been become an important livelihood activity of farm households in Vietnam, especially coffee and pepper production in the Central Highlands region. In 2015, Vietnam had 2,486 thousand hectares of perennial industrial crops, of which coffee and pepper contributed about 30% of the total perennial crop area (GSO, 2016). In Dak Lak province, traditional farming practices of coffee and pepper have now been expanded from a mono-crop system (separating coffee and pepper) to an intercropping system, where coffee and pepper are grown together. This system is considered to have high economic potential (Institute of Engineering Sciences and Agriculture - Forest Highlands, 2011) and is considered to be a means for farmers to escape from poverty (Huy Hoang, 2014). CDC (2013) also mentions the advantages and disadvantages of intercropping coffee with pepper. In the Quang Hiep commune, coffee and pepper accounted for nearly half the total natural area in 2014, coffee and pepper intercropping (CPIC) has been practiced since 1999, and has been reported to play an important role in improving socio-economic status in the commune. It is believed that the expansion of the system is generally spontaneous where farmers work by their own experiences in intercropping coffee and pepper. This study was designed to evaluate the current economic performance and factors affecting economic performance of CPIC in Quang Hiep commune, to provide information for agricultural managements, extension centers as well as the local authority so that they can orient and help to develop this intercropping system in the future.

2. RESEARCH METHODS

2.1. Data collection

The secondary data relevant to CPIC in Quang Hiep commune, CuMgar district, Dak Lak province was gathered from the statistical Yearbook of Dak Lak province between 2012 and 2014, annual reports included: province, district and commune, and Provincial People's Communities. Other reports from books, newspapers, websites, and previous studies or thesis reports of similar topics were also collected.

Primary data was collected through surveys of 50 CPIC households, randomly stratified by production area, as normally differentiated by local people, which were classified into three groups of small (< 1 ha), medium (1 - 3 ha), and large (> 3 ha), as suggested by extension workers. In-depth interviews with the leaders of the commune were conducted with commune and village leaders, extension workers and 10 collectors who buy coffee and pepper from farmers.

2.2. Data analysis

Descriptive statistical analysis was applied with simple statistics such as means and growth rate, with the aids of tables and charts. Comparative statistics were employed with simple t-tests and F-tests for means comparison. The major criteria for financial analysis for farm households were costs, value added, and net farm income (EC, 1989; Farm Financial standards Council, 1997).

3. RESULTS AND DISCUSSIONS

3.1. Coffee and pepper intercropping in Quang Hiep commune

Intercropping is the growing of two or more crops simultaneously in the same field. The practice of relay intercropping involves planting a second crop after an initial crop has reached maturity, but before it is ready for harvest. According to Larry and Barbara (2001), one application of relay intercropping is to divide crops into two categories: the main component is the crop of primary importance and has the desired yields; the second crop, or secondary component, provides added economic and/or environmental benefits. The polyculture (multi cropping/ intercropping) is used commonly in agriculture. Ofori and Stern (1987) suggest that

growing two or more crops simultaneously is more efficient than monocropping for exploitation of limited resources. However, a major concern in using intercropping systems on infertile soils is the accelerated depletion of mineral nutrients when both crops are harvested. Coolman and Hoyt (1993) highlighted that when overlapping crops in space and time, the growth of two or more crops often results in decreased yields of both crops due to competition for limited essential resources. Any development of intercropping systems must evaluate the effects of competition on crop yields.

Coffee and pepper intercropping was first practiced in Quang Hiep commune in 1999 by a chairman of the commune, who also shared his experiences with other farmers. The intercropping area expanded quickly and reached 450 ha in 2014 (Table 1), accounting for about 18% of the total coffee and pepper areas in the commune. The total output production of pepper has increased significantly, from 742 tons to about 1400 tons during 2012 - 2014, where coffee production exhibited an unstable trend, with decreased volume in 2014, due to reductions in both area and yield.

3.2. Economic performance of coffee and pepper production in farm households

3.2.1. General information on farm households and the intercropping system

About three-fourths of the interviewed households are headed by men, with an average age of about 42 years old and 9 years of schooling (Table 2). On average, a household has 2.1 ha of cultivated land, of which the intercropping area is 1.5 ha. Almost all households have pumps and wells for coffee and pepper production, largely thanks to a national grip program in the commune.

Table 1. Selected indicators of coffee and pepper production in Quang Hiep commune (2012 - 2014)

Indicators	2012	2013	2014	Comparison (%)	
				2013/2012	2014/2013
1.Total coffee and pepper area	2,259.5	2,340.5	2,540	103.58	108.52
Of which, intercropping area	225	335.5	450	149.11	134.13
2. Total production (tons)					
Coffee	5,490	6,985	6,680	127.22	95.63
Pepper	742	994	1,423	133.91	143.22

Sources: Statistics from Quang Hiep commune (2015)

Table 2. Characteristics of CPIC households in Quang Hiep commune

Indicators	Value
% households headed by men	76
Age of household heads (year)	41.5
Number of schooling years of household heads (year)	9
Experience with CPIC of households (year)	7
Total cultivated land area per household (ha)	2.1
CPIC area per household (ha)	1.5
Production capital per household (million VND)	165
Labor working in coffee and pepper production per household (people)	2.3

Source: Calculated from household survey, 2015

Table 3. Characteristics of CPIC gardens in farm households, by production scale

Items	Small (n = 14)	Medium (n = 31)	Large (n = 5)
1. Method (% households)			
Group	8	30	2
Intersection	20	32	8
2. Density (trees/ha)			
Coffee	950 - 1,000	900 - 1,000	900 - 950
Pepper	700 - 750	650 - 700	650 - 700
3. Age of intercropping garden (% household)			
Under 5 years	16	18	2
From 5 - 15 years	6	26	8
Over 15 years	6	18	0

Source: Calculated from household survey, 2015

Generally, two methods of intercropping coffee and pepper are now practiced in Quang Hiep commune, namely the group and intersection methods. The intersection method is a way that produces plants in 2 - 3 coffee rows to intercrop one pepper row (the pepper crop is cultivated at the intersection point of the coffee holes), where in the group method, small sub-areas of coffee and pepper are designed in the garden. Among interviewed households, large and small ones tend to choose the intersection method more often, where the medium sized households balanced between the two methods (Table 3).

Cropping density varied from about 900 - 1,000 coffee trees/ha and 650 - 750 pepper trees/ha (Table 3), however, small households tended to have a higher density, for example, reaching a maximum of 1000 coffee trees/ha and 750 pepper trees/ha whereas the large ones practiced lower density (Table 3), which is better, according to the commune extension worker. Nearly half of farm households have intercrop gardens aged from 5-15 years.

3.2.2. Production costs

Production costs of the intercropping garden were decomposed into two types, the depreciation cost of fixed assets and variable costs. Large households incurred the highest production costs, estimated at about 47 million VND/ha for the crop year 2014 - 2015 (Table 4). Fertilizer accounted for the largest part of production costs, at about 50% for all households. Medium and large households generally applied more fertilizer and water for their gardens.

Table 4. Production costs of coffee and pepper intercropping system, by production scale

Items	Farm size			All
	Small	Medium	Large	
1. Variable costs	37.1	41.1	41.6	40.1
Fertilizer	20.1	23.0	23.6	22.3
Watering	0.8	1.2	1.2	1.1
Pesticide	0.6	0.9	0.9	0.8
Hired labor	15.5	15.9	15.8	15.8
Others	0.1	0.1	0.1	0.1
2. Fixed costs	5.3	5.4	5.5	5.3
Total cost	42.3	46.5	47.1	45.4

Source: Calculated from household survey, 2015

Table 5. The average yields and total production of intercropping based on farm sizes

Indicator	Farm size			Average
	Small	Medium	Large	
1. Yield (tons/ha)				
Coffee	2.7	2.75	3	2.76
Pepper	1.47	1.52	1.55	1.51
2. Total productivity (tons/household)				
Coffee	1.43	4.5	10.1	4.20
Pepper	0.78	2.5	5.4	2.31

Source: Calculated from household survey, 2015

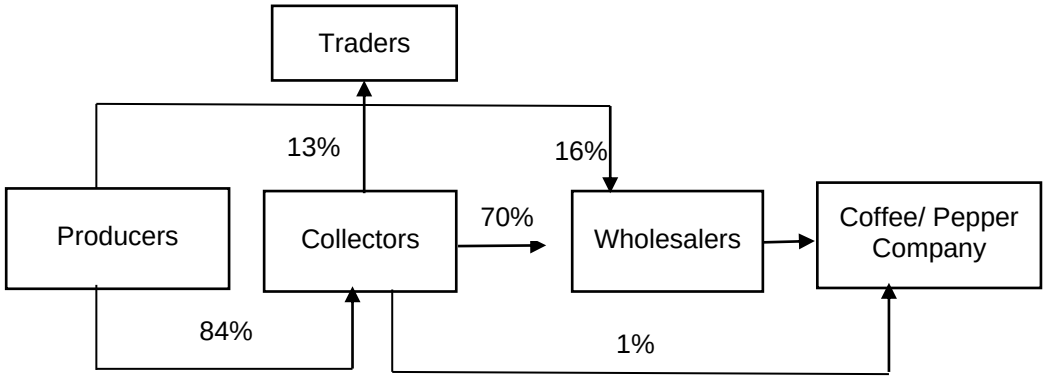

Figure 1. Marketing channel of coffee production of farm households in Quang Hiep commune

Sources: Household survey, in-depth interviews with collectors, wholesalers, 2015

3.2.3. Yield, production volume, and marketing of coffee and pepper

On average, a hectare of coffee and pepper intercropping produces 2.76 tons of coffee and 1.51 tons of pepper (Table 5). Large households achieved the highest yields, estimated at about 3 tons of coffee and 1.6 tons of pepper, largely thanks to higher levels of investments and more careful seed sourcing. Large farms were also found to often update information about diseases, quality fertilizers, and the best pesticides, and consult with local plant protection experts in cases of disease. With higher production areas and yields, large-scale farm households attained the highest volumes of production, with averages of about 10 tons of coffee and 5.4 tons of pepper in the 2014 - 2015 crop year (Table 5).

Almost all coffee and pepper produced (84%) go to local collectors, who reside in the commune and buy coffee and pepper from households to sell to wholesalers and traders. Most of the traders are located outside of the commune. There are two coffee companies, namely D'rao and Ea Pok ones, located in other communes and at the district center, but there is no pepper company in CuMgar district. Farmers prefer to sell their coffee and pepper to collectors in order to save transportation costs and get cash quickly, even if they sell at lower prices. In-depth interviews with village heads reveal that there are about 1-2 collectors in each village, which is convenient for farmers in selling their products.

About 16% of coffee production goes to wholesalers and comes mostly from larger farms with considerably higher production volumes

and higher quality (humidity, foreign matter, etc.), which is valued higher by wholesalers.

3.2.4. Economic performance of coffee and pepper intercropping

On average, a hectare of coffee and pepper intercropping generates a total value of about 374 million VND with 216 million VND of value added during the 2014 - 2015 crop year (Table 6). Larger production scales seem to generate higher economic performances in coffee and pepper intercropping, with total net family income/ha of large farm households estimated at about 237.8 million VND/ha, much higher the figures from small ones (Table 6).

In comparing the economic performances between the monocropping systems (i.e. coffee or pepper separately), it was shown that the intercropping system had a higher performance in terms of total revenues, value added, and net farm income. For example, net farm income from 1 ha of an intercropping system was estimated at about 202.7 million VND, much higher in comparison to a mono coffee cropping system (142 Million VND) and a mono pepper cropping system (48.8 million VND/ha) (Table 7).

3.3. Factors affecting the economic performance of coffee and pepper production

Infrastructure, crop disease, input and output prices, and weather are considered to be the most common factors that negatively affect crop production, as reported by all farmers (Figure 2). From data analysis, it was also found that the production scale and age of trees also influence the economic performance of the CPIC.

Infrastructure

Infrastructure was reported as one of the most important factors affecting coffee and pepper production, especially in terms of roads to transport coffee, electricity, and irrigation systems. Water was reported by about 54% of farmers as one of the impediments to coffee and pepper yields in the commune (Figure 2), especially during the dry season. Farmers have to use pumps to get water from wells, but in many cases three-phase electricity wire was not available, and the use of gasoline was also expensive. Coffee and pepper yields were shown to be statistically different between being watered and not (Table 8). As a result, higher NFI/IC ratios were seen in the gardens being watered. This also coincided with findings from Cheesman and Bennett (2015).

At the significant level of 5%, the test results illustrate that $t_{obs} > t_{crit}$. The alternative hypothesis, H_1 is accepted while the H_0 rejected. This means that the performance indicators are not the same between households with training and households without training. When the household heads are not trained, they do not apply modern techniques in production such as fertilizers, planting design, and disease prevention. They just implement by their experiences. Therefore, the yields of coffee and pepper are low.

Table 6. Economic performances of coffee and pepper intercropping, by production scale

Indicator	Unit	Farm size			Average
		Small	Medium	Large	
Total revenue (TR)	Mil. VND	355.2	375.2	415	373.6
Intermediate cost (IC)	Mil. VND	154.2	158.4	161.2	157.5
Value added (VA)	Mil. VND	201.0	216.8	253.8	216.1
Net farm Income (NFI)	Mil. VND	191.0	202.3	237.8	202.7
TR/IC		2.16	2.17	2.34	2.2
NFI/IC		1.24	1.28	1.48	1.3

Source: Calculated from household survey, 2015

Table 7. The economic performances of coffee and pepper by production system (per ha)

Indicator	Farm type		
	Only coffee	Only pepper	Intercropping
TR (Mil. VND)	128.5	288	373.58
IC (Mil. VND)	68	135.25	157.50
VA (Mil. VND)	60.5	152.75	216.08
NFI (Mil. VND)	48.8	141.75	202.69
TR/IC	1.61	1.95	2.18
NFI/IC	0.72	1.05	1.29

Source: Calculated from household survey, 2015

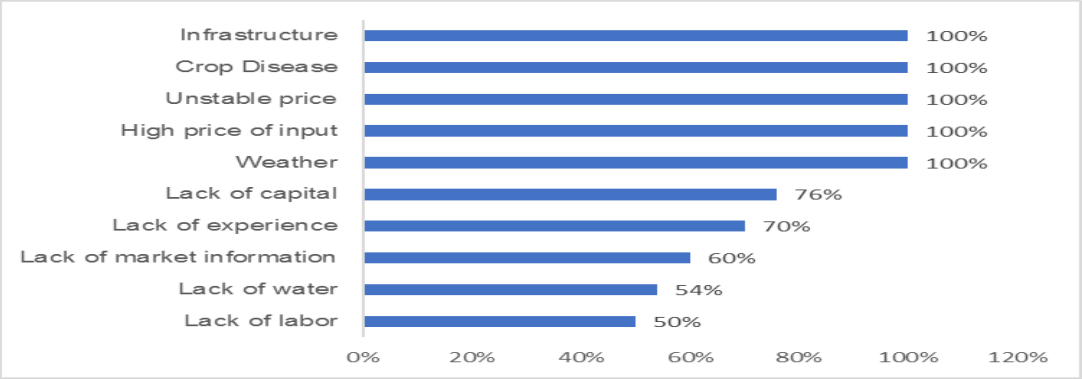

Figure 2. Constraints in coffee and pepper production, as perceived by farmers

Source: Calculated from household survey, 2015

Table 8. Test results of selected factors on coffee and pepper yields of intercropping system performance

Factors	Coffee yield	Pepper yield	NFI/IC
Irrigation			
Crop is watered	2.8	1.52	1.21
Crop is not watered	2.59	1.4	0.85
Difference	0.21**	0.12***	0.36***
Production scale			
Small	2.7	1.47	1.0
Medium	2.8	1.52	1.26
Large	3	1.55	1.5
F-stat	8.4***	7.03***	26.6***
Seed selection			
Bought from seed company/center	2.94	1.53	1.36
Produced by farmers	2.7	1.48	1.1
Difference	0.24***	0.05*	0.26***
Training of famers			
Farmers were trained	2.85	1.54	1.35
Farmers were not trained	2.72	1.48	1.09
Difference	0.13**	0.06**	0.26***

*Note: *, **, ***: significant at 10%, 5%, 1%, respectively*
Source: Calculated from household survey, 2015

Production scale

A larger production scale was found to have a better economic performance than a smaller scale (Table 8) for saving production costs, especially labor costs in caring and harvesting. Moreover, the large-scale farmers often use better techniques and good machines so that labor is saved, and their gardens tend to be attacked by diseases and pests less often than in small gardens. It was revealed during the survey that small scale farmers often do not invest carefully and they reasoned that gross output from coffee and pepper was not very significant. They did not have much money to invest in their farms, but if their production area expanded in the future, they would invest more.

Crop disease and insects, and weather

Serious crop diseases and pests such as coffee rust, Coccus viridis (Green), cicada infestations, nematodes (yellow leaf), mealy bugs, and stem borers caused yield damage. No data was available to show the negative impact of crop diseases and pests on crop yield but farmers expressed their serious concerns on the matter. Farmers reported that they could still manage these problems but it was difficult, especially with aging crops, degraded soil, and abnormal weather (drought and erratic rain). Abnormal weather was also reported to contribute to crop diseases by all farmers (Figure 2). At the time of the study, there was no plant protection specialist in the commune and farmers had to go to the district to consult with extension specialist when the crops got diseases.

Inputs

Coffee and pepper producers in Quang Hiep commune are heavily reliant on fertilizer usage and gasoline. These input prices are high and volatility was cited as a major concern by the farmers. Among inputs, seed quality was cited as an issue in production. About 90% of farmers either bought coffee and pepper seeds from other households or produced the seeds themselves, and the selection of seeds is based on only size (big) and appearance (looks good, no scratches, no evidence of pests/insects, good color), without knowing the seed quality. About 10% of farmers bought seed from the seed center, seed company, or EaKmart institution, where seed is selected quite carefully. As a result, seed bought from seed a company/center provided higher yields (Table 8).

Farmers' knowledge and expertise in production

Coffee and pepper intercropping is still a new technique to Quang Hiep farmers, and knowledge about planning, caring for, and harvesting is required. A training course on coffee and pepper sustainable production was held in 2014, and farmers were also trained four times about technical planning, caring for, using fertilizers, using pesticides, harvesting and processing. Some companies such as Viet Nhat Fertilizer Cooperation, Binh Dien, Hoa Cuong, Nhat Loc Phat provide trial products and share their experience with farmers. According to the survey data, just about 50% of farmers participated in the trainings. Testing results also showed that the economic performance of the CPIC was different among farmers who attended training and those who did not (Table 8). It was also observed that trained farmers usually apply the intersection method, while non-trained farmers applied the method of intercropping coffee and pepper. According to the survey data, 24% of farm households harvested coffee where the rate of ripened berries was under 50%

Output prices and information

Unstable price of coffee beans is probably one of the largest concerns of farmers, which varied over years and seasons. Two-thirds of farmers did not have full information on the coffee market, mostly depending on local traders. Information from the internet, as reported, had limited value to farmers, as they depend on local traders who buy coffee from farmers. No formal linkage between farmers and traders/buyers was found among the farmers.

Gender, ethnicity, and others

About three-fourths of farm households are headed by men. Coffee and pepper production is

usually considered to have heavy tasks (land preparation, watering, caring, harvesting, etc.). Machines and other equipment (pumps, transportation vehicles, and others) used in production also require strength that women may lack so male- headed households are expected to have better performance in production. It was revealed from interviews with the extension workers that most participants attending trainings are male, which was also confirmed by the test results (Table 9). It is worth noting that ethnicity also has implication to economic performance in production, with Kinh farmers having better economic performance than other farm households (Table 9).

Farmers also reported that they lacked capital, which constrained them in investing in their garden or forced them to borrow from an informal financial system with high interest rates. Crop age also influenced productivity, with peaks attained at about 12-13 years for pepper and 13-15 years for coffee (Figure 3). This suggests that famers might need to improve their techniques to slow down the decreasing rate of yields after peak years.

3.4. Recommendations for improvement of economic performance of coffee and pepper intercropping in Quang Hiep Commune

Production plan of coffee and pepper intercropping system in the commune

Land for production is limited in Quang Hiep commune, so the intercropping of coffee could be a solution. Leaders in Quang Hiep commune have developed a master plan for coffee and pepper production to the year 2020, by which the total area of coffee and pepper intercropping is projected to be about 450 ha in 2016 and 460 ha in 2020. The plan is not only based on available area for production, but also depends on market conditions.

Table 9. Differences in coffee and pepper intercropping performance by gender and ethnicity

Factors	Coffee yield	Pepper yield	NFI/IC
Gender of household head			
Male	2.81	1.55	1.23
Female	2.67	1.44	0.9
Difference	0.14**	0.11*	0.33***
Ethnicity			
Kinh	2.8	1.51	1.22
Others	2.6	1.44	0.92
Differences	*0.2***	*0.07**	*0.3****

*Note: *, **, ***: significant at10%, 5%, and 1%, respectively*
Source: Calculated from household survey (2015)

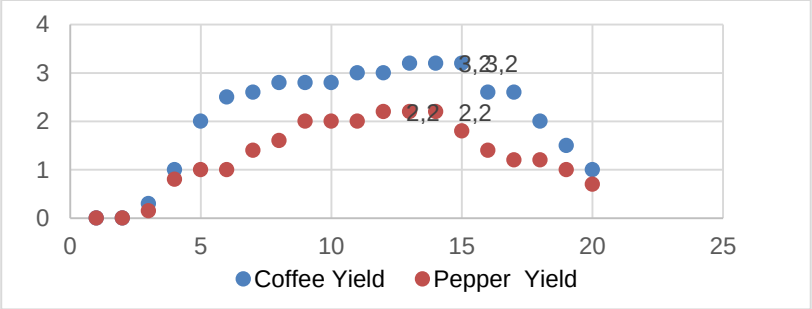

Figure 3. The yields of coffee and pepper products in intercropping by year

Sources: Calculated from data, 2015

Table 10. The Master Plan of Quang Hiep commune for the period of 2016 - 2020

No	Items	2016	2020
1	Perennial industrial crop area (ha)	2,572	2,630
	Coffee and pepper intercropping (ha)	450	460
2	Yield (ton/ha)		-
	Coffee	3.2	3.5
	Pepper	2.4	3.2

Source: Calculated from household surve, 2015

Recommendations

Improving farmers' knowledge and techniques on intercropping coffee and pepper: As mentioned above, intercropping is new to farmers and most of them practice based on their own knowledge. Therefore, they often lack the knowledge and skills in planting, caring for, and harvesting their crops. Future training should focus on the construction period (plant density and methods of intercropping), and the harvesting period (using fertilizer, detecting insects and disease prevention, harvesting methods (e.g. having a rate of over 80% ripened berries in order to improve product price and value), as well as preventive measures against crop diseases and insects, especially for pepper). Trainings also should focus more on female and ethnic farmers.

Increasing investment and encouraging use of quality seed: Economic performance of CPIC is greatly influenced by seed quality. Hence, local authorities should create good conditions for farmers to approach and use new, quality seed. Also, technical guidance on seed selection for intercropping should be provided. At the time of surveying, there was no seed provider inside the commune, so private seed providers should be encouraged to set up. During training, recommendations on seed selection as well as encouraging farmers to use quality and certified seed should be addressed. Pillars for pepper should replace timber pillars for higher effectiveness and lower costs, as well as exhausting timer sources.

Infrastructure improvement and other supports from local government and line agencies: Public investment in basic infrastructure, especially irrigation systems, is recommended. Local authorities might practice activities to support farmers in finding water sources, or develop measures to save water in the dry season. Extension services, as the abovementioned, should focus more on the intercropping system. The local government could also be an intermediate in setting up linkages between farmers and buyers in order to mitigate market risks for farmers. Coffee prices, other input prices, and other market information should be designed and disseminated effectively and efficiently to farmers.

Improve farmers' capability in production and negotiation with traders: At the time of surveying, crop production at farm households faced a number of difficulties because the high costs as well as the low product prices. Small farms with low volumes of production usually have to sell at lower prices. Therefore, improving the production capacity (land and capital) and the negotiation capability for farmers are necessary. One possible solution is to set up a region for coffee and pepper intercropping (e.g. probably more than 20 ha) such that the volume is high enough to get better prices. Farmers might be organized into groups to have a better voice in negotiating output and input prices, especially to escape the price squeeze of collectors or traders.

4. CONCLUSION

The coffee and pepper intercropping system has been practiced widely in Quang Hiep

commune with rapid increases in terms of area, reaching 450 hectares in 2014, producing about 6,680 tons of coffee and 1,423 tons of pepper. The system has become a key cropping system for local economic development. The study showed that the average area of intercropping reached 1.5 ha/ farm household. On average, income returned to household was estimated at about 203 million VND/ha in the 2014-2015 crop season. There was also evidence that the intercropping system is more economically efficient than the mono-system. The economic performance of the intercropping system was found to be different between households and depended on production scale, irrigation status, seed quality, gender, ethnicity. and the farmers' knowledge. The production system now faces other challenges, such as a lack of water resources, farmers' technical know-how, crop diseases and pests, changing weather, finances, and market information.

We proposed a set of recommendations to improve economic performance of the coffee-pepper intercropping in Quang Hiep commune, namely improving the farmers' knowledge and techniques on intercropping of coffee and pepper, increasing investments, encouraging the use of quality seed, improving infrastructure and other support from local government and line agencies, and improving the farmers' capability in production and negotiation with traders. And there is a need to conduct a research on the effectiveness and economic performance of the system over a longer span of time. Advantages and disadvantage as well as potentials to expand the system should also be further studied.

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DEVELOPING CORN VALUE CHAINS OF MINORITY ETHNIC HOUSEHOLDS IN LAO CAI PROVINCE, VIETNAM

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Received date: 21.03.2017 Accepted date: 11.09.2017

ABSTRACT

This study focused on analyzing corn value chains in Lao Cai province as well as proposing solutions to upgrade the corn value chains in order to improve the livelihoods of ethnic minorities in Lao Cai. The study used data from a survey of 120 corn ethnic minority households and corn value chain actors in Lao Cai in 2016. Descriptive statistics, comparative statistics, and the values chain analysis method are the main methods used in the study. Research results showed that farmers used outdated cultivation techniques, especially ethnic minority households in upland communes, and often produced corn by extensive farming methods (no fertilizer or a small quantity of fertilizer) with unsecured technical procedures. The product distribution channel is long with many stakeholders. Nearly 75% of the corn producers sell their products to traders in their commune who then sell it to three other levels of agents (the district, province) before the products reach the consumer.

Keywords: Corn value chain, ethnic minorities, livelihoods.

Phát triển chuỗi giá trị ngô của các hộ dân tộc thiểu số tại tỉnh Lào Cai, Việt Nam

TÓM TẮT

Nghiên cứu tập trung phân tích chuỗi giá trị ngô tại tại tỉnh Lào Cai cũng như đề xuất một số giải pháp nâng cấp chuỗi giá trị ngô từ đó nâng cao sinh kế cho đồng bào dân tộc thiểu số ở Lào Cai. Nghiên cứu sử dụng số liệu điều tra 120 hộ đồng bào dân tộc thiểu số trồng ngô và các tác nhân tham gia chuỗi giá trị ngô tại Lào Cai năm 2016. Các phương pháp thống kê mô tả, thống kê so sánh, phân tích chuỗi giá trị là các phương pháp chính sử dụng trong nghiên cứu. Kết quả nghiên cứu cho thấy Sự hiểu biết không đồng đều và thấp, đặc biệt là các hộ gia đình dân tộc thiểu số ở các xã miền núi thường sản xuất ngô theo các phương pháp canh tác rộng rãi (không phân bón hoặc phân bón rất ít) với các quy trình kỹ thuật không đảm bảo. Kênh phân phối sản phẩm dài với nhiều bên liên quan. Gần 75% hộ sản xuất ngô bán sản phẩm của họ cho các thương nhân tại xã của họ và sau đó bán lại cho 3 tác nhân khác (quận, huyện, tỉnh) trước khi đến với người tiêu dùng.

Từ khóa: Chuỗi giá trị ngô, dân tộc thiểu số, sinh kế.

1. INTRODUCTION

Corn is one of the key agricultural products in Lao Cai province. According to statistics in 2015, corn planted acreage in Lao Cai province was approximately 37,434 hectares, the average corn yield was over 3.6 tons/ha, and output was about 133,152 tones/year (Lao Cai Statistic Department, 2016). However, the circulation of corn products is difficult. The linkage between producers and other stakeholders in the value chain is not tight. The main consumers of corn products in Lao Cai are Chinese, which may lead to fluctuations in prices, and low and unsustainable income for corn farmers, greatly affecting the livelihoods and living conditions for people in this region. Therefore, the development of the corn value chain in Lao Cai

province still faces many difficulties. The main objective of this study was to assess the market performance of the stakeholders in the hybrid corn value chain, propose strategies to improve the economic value of the chain, and increase income for producers and other stakeholders in the chain, especially for the poor and ethnic minority households in Lao Cai province.

2. DATA AND RESEARCH METHODS

2.1. Methods of data collection

Secondary data: Data on production and consumption of the products was collected from statistics of the districts and province. Reports were collected on the production, processing, and consumption of hybrid corn in the province, districts, and communes within the study sites. The studies were related to the value chains for agricultural products from various sources.

Primary data: Primary data was collected through direct interviews with households involved in the production and trading of corn with using contents in a questionnaire (semi-structural) prepared in advance and with additional questions for clarification. For this study, 120 corn farmers were randomly selected and interviewed in three different districts of Lao Cai province (Bac Ha, Muong Khuong and Simacai). These districts have the largest number of hybrid corn producers and the highest quantity of hybrid corn in Lao Cai. In addition, collectors, processors, and other traders involved in the value chain of hybrid corn in the communes and districts were interviewed using prepared questionnaires.

Key person interview (KPI): In addition, the leaders of the relevant departments (DARD, DOIT, Agriculture Extension Center), leaders of the districts, and leaders of the professional departments in the three surveyed districts and villages were interviewed to collect information about the overall corn production status of the province, districts, and communes. The information collected from those interviews helped us create an overview of development strategies for corn production in Lao Cai province.

Focus group discussions: From each village, one group of 5-10 people acted as representatives for their economic circumstances, gender, and ethnicity, and was invited to the village cultural building or village leaders' houses to collect information. The methods of participatory discussions were made during the group meetings to collect information on the production and consumption of corn in the villages, in addition to the social and cultural elements, experiences, production, marketing of products, and price fluctuations from many different perspectives.

2.2. Data analysis

Data collected from the stakeholders involved in the value chain of corn was input into Excel and processed using STATA software.

The study used descriptive methods, comparative statistics methods, and value chain analysis methods to describe the status of production, processing, and consumption of products in the corn value chain in Lao Cai province. These methods also helped analyze and compare the distribution of benefits and costs among stakeholders in the hybrid corn value chain.

SWOT matrix analysis was also conducted to assess the internal and external factors, including strengths, weaknesses, opportunities, and threats/risks, that are affecting the development of the hybrid corn value chain. Therefore, this analysis helped us propose solutions/development strategies to upgrade the value chain of hybrid corn.

3. RESULTS AND DISCUSSION

3.1. Analysis of the hybrid corn value chain

3.1.1. General information on corn production in Lao Cai province and the surveyed districts

Lao Cai is a mountainous, border province with three sides bounded by Yen Bai, Ha Giang, and Lai Chau provinces, and one side bordering Yunnan Province (China). Corn is one of the

main agricultural crops of Lao Cai. Lao Cai province had a total corn acreage of 37,434 hectares, with the average corn productivity of over 3.6 tones/ha and a yield of 133,152 tones. More than 90% of the total area is planted with hybrid corn, when the rest (less than 10%) is for waxy corn and other local corn varieties (Lao Cai Department of Agriculture and Rural Development, 2016).

Muong Khuong, Simacai, and Bac Ha are three mountainous districts that have large corn acreage in Lao Cai province, accounting for 19.05%, 12.15% and, 15.24% (over 46%), respectively, of corn acreage in the province in 2015 (Lao Cai Department of statistics, 2017). The total production of corn in 2015 in Lao Cai province was 133 thousand tons, while corn production in the three districts of Muong Khuong, Bac Ha, and Simacai contributed over 45% of the total corn output of Lao Cai province (Lao Cai Department of statistics, 2017).

Corn yields in Lao Cai province in general and districts in particular are still low, around 3.5 tones/ ha. With favorable weather and climate, Lao Cai province in general and the three districts of Muong Khuong, Bac Ha, and Simacai plant corn in three major seasons as follows:

- Spring corn crop: Plant in February - March in lowland districts/communes

- Summer corn crop: Plant in March - April in upland districts/communes

- Summer-autumn corn crop (main crop): Plant in the end of June - middle of July in upland districts/communes and alluvial land in lowland areas.

The procedures for planting and growing corn are simple, so they are suitable for intensive levels by ethnic minorities. Currently, the majority of the people already know how to apply technological advances to production (the rate of hybrid corn used in production in the localities occupies more than 90% of the cultivated area), thereby improving productivity, output, and economic efficiency of corn production. However, a selection of the people from ethnic minorities and poor households who lack funds and scientific and technical knowledge are cultivating corn in the form of extensive planting (using less fertilizer), and abusing plant protection products (mainly herbicides).

Table 1. Acreage, productivity, and yield of corn in Lao Cai province and surveyed districts, 2015

		Unit	Lao Cai province	By district		
				Muong Khuong	Simacai	Bac Ha
Yearly average	Acreage	ha	37,434.0	7,130.0	4,550.0	5,706.0
	Productivity	quintal/ha	35.6	34.1	34.6	34.9
	Yield	ton	133,152.0	24,325.0	15,727.0	19,918.0
In which:						
1. Winter corn	Acreage	ha	806.5	-	-	-
	Productivity	ton/ha	2.81	-	-	-
	Yield	ton	2,266.0	-	-	-
2. Spring corn	Acreage	ha	11,318.0	1,530.0	100.0	338.0
	Productivity	ton/ha	3.65	33.4	28.5	28.2
	Yield	ton	41,337.0	5,110.0	285.0	953.0
3. Summer-autumn corn (main season)	Acreage	ha	12,618.0	4,000.0	3,250.0	3,918.0
	Productivity	ton/ha	3.56	35.2	35.3	36.6
	Yield	ton	44,923.0	14,065.0	11,458.0	14,340.0
4. Spring-summer corn	Acreage	ha	12,691.0	1,600.0	1,200.0	1,450.0
	Productivity	ton/ha	3.52	32.2	33.2	31.9
	Yield	ton	44,626.0	5,150.0	3,984.0	4,625.0

Source: Lao Cai Provincial Statistics Office, 2016

3.1.2. Description of the corn value chain

The average planted area with hybrid varieties in 2015 accounted for 97.5% of the total corn planted area. Therefore, in this study, we focused on the value chain analysis of the hybrid corn produced by corn farmers in the studied areas.

As can be seen in the diagram of the corn value chain of the three studied districts (Figure 3.1), there are six main stakeholders in the corn value chain, namely (1) Input suppliers; (2) Corn producers; (3) Village/Commune collectors; (4) District-level collectors; (5) Province-level collectors (companies, agricultural product processing and export enterprises); and (6) Retailers.

Corn marketing channels: The value chain diagram of corn shows that there are two main marketing channels (traditional) occupying the largest proportion of the commercial corn in the studied districts, including:

Marketing channel 1: Corn farmers ⇨ Village/commune collectors ⇨ District-level collectors ⇨ Retailers ⇨ Consumers

The survey showed that most of the corn producers use about 25% of the total hybrid corn production for animal feeds and cooking wine, while 75% is for commercial purposes. About 40% of corn production in the chain is collected by the collectors at the communes and 20% by households who have large-scale production to sell directly to the major collectors in the district. The mobile collectors sell about 36% of the products purchased to district-level collectors and 4% to grocery shops in the communes. The grocery shops in the villages sell about 16.15% of the products purchased to district-level collectors and 2.85% of corn production to the provincial collectors (large firms of agribusiness processors, such as An Nghiep Company Limited, Anh Kien Company Limited, and Tay Bac Import Export Company Limited). After collecting corn from the communes, district-level collectors conduct preliminary processing and packaging, and sell about 10.85% to traders in southern provinces (Ho Chi Minh City, An Giang, Dong Nai, etc.) and the northern provinces (Hanoi, Hai Duong, Hung Yen, etc.), about 50.5% to large province-level traders and enterprises, and 3.6% to the millers and retailers in the districts. The large collectors in the province (companies and agribusinesses) export around 34.68% to China, and sell 18.67% to traders in the southern and northern provinces.

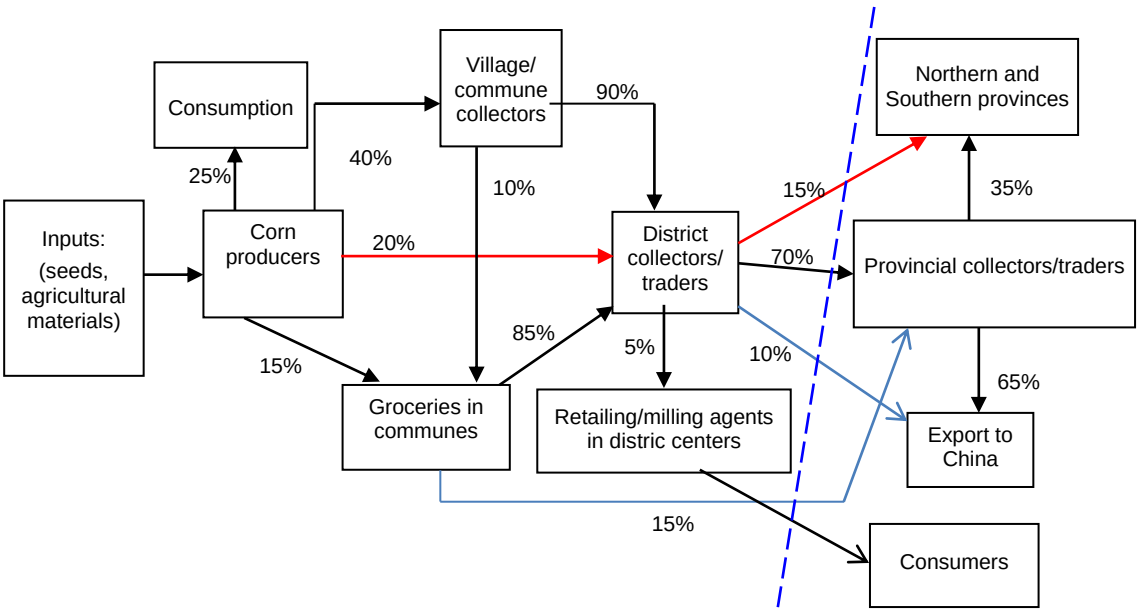

Figure 1. The hybrid corn value chain of the surveyed districts, Lao Cai province

Besides the aforementioned traditional hybrid corn consumption marketing channels, in the surveyed areas, there are five others hybrid corn marketing channels including (1) Marketing channel 2: farmers sold hybrid corn to district level collectors. Then retailers bought hybrid corn from district level collectors after that they sold it to consumers. The third channel, it was similar with channel second, however district level collectors did not involve in this channel. The fourth channel was different with previous channel, district level collectors bought hybrid corn from farmers, then they sold to province level collectors. Province level collectors export hybrid corn to China. Fifth channel was similar with the fourth channel, however famers sold directly hybrid corn to province level collectors. The final channel was simple, famers sold directly hybrid corn to consumers.

Although some marketing channels bring higher profits for stakeholders than marketing channels 1 and 2, due to capacity constraints and the stakeholders (collectors) being out of the surveyed areas (channels 3 and 4), or low proportion of corn commodities exchanged in these channels (channels 5 and 6), we could not approach or analyze them in depth.

3.1.3. Analysis of the stakeholders in the corn value chain

3.1.3.1. Input suppliers

There are two business modes that input suppliers for corn production in Lao Cai. The first type includes level 1 and level 2 agents specialized in selling agricultural materials and seed at district centers or clusters of communes. The second type is the grocery shops in communes selling agricultural materials and purchasing agricultural products (corn, rice, etc.). About 70.5% of the corn producing households buy materials and seeds from grocery shops. Several corn farmers said that it is easier to purchase seeds and materials from the grocery shops and to pay on credit. Moreover, they buy a small amount but many times, so they think that buying in the grocery shops in the communes is more favorable.

Most of the input suppliers must borrow money, of which a high proportion of the money borrowed is from banks, and about 65% of input suppliers use loan money to ensure they have enough working capital. However, input suppliers usually have difficulty accessing loans from banks to get enough money to serve their business.

3.1.3.2. Producers

* General characteristics of the surveyed households

Information collected from the surveyed corn household farmers in communes of the three studied districts are shown in Table 3.3.

The average age of household heads of corn farmers in the surveyed areas was 41 years old, the figure in Simacai district was the lowest (37 years old). The lower age of the household heads in corn producing households is one of the advantages in transferring advanced technologies in corn production because they can approach the knowledge more easily and readily. However, the literacy of corn farmers is quite low with 4.3 years of formal education on average. This will be one of the limitations and challenges for accessing market knowledge, negotiating, and applying technological advances in corn production. The number of main labors / household in the surveyed districts ranges from 1- 6 people, with an average of 3.1 people / household. The total average income of the corn producing households ranges from 14.7- 54.9 million VND/year. The income from corn production contributes approximately 21.7 - 28.4% of the total income of the household groups in the surveyed districts, and the average for all of the surveyed corn producing households is 24.3%. This means that corn plays an important role in improving income of the households in the areas.

The average number of years of corn planting experience of corn farmers is 18.6 years, while households in Muong Khuong have the most years of experience in corn planting (20.2 years). Most corn producing households lack capital, and 90.2% of surveyed households said that they are in lack of capital for production.

* Status of corn production of the surveyed households

The average agricultural acreage of households is 0.9 ha/household while corn planted acreages of households is approximately 0.6 ha/household. The average number of corn planting plots per household reached around 2. With the adoption of policies on concentrating farming land for large production areas within the province and districts, the fragmentation of land for agricultural production has improved significantly. However, there are still a number of areas with households owning 4-5 plots, and the fact that corn plots of the households are scattered will be one of the obstacles for cultivation, and increases the cost of production, harvesting, and selling products. Corn productivity on average for the surveyed households reached 3.8 tons/ha, and this corn productivity is still higher than in Lao Cai with an average of 3.4 ton/ha (Tran Hoang, 2015).

* The status of corn consumption of the households

The buyers of corn from the households: Due to far distances from households to district centers, they usually sell a large part of their corn product to collectors in the villages/communes by exchanging corn for other living necessities or agricultural materials (approximately 55% of corn yield produced by the households). Meanwhile, corn producers in the communes near the district centers, who have large yields of corn and vehicles, are more likely to sell corn to large district-level collectors (20%) at higher prices than those selling to commune-level collectors. Most of the households sold dried corn after it was harvested. The main reason is that households can store dried corn and sell it when the selling price is high or when cash is needed.

In terms of negotiation ability on the selling price of farmers, if they are able to negotiate and make decisions about the price of the product, they will have more opportunities to get more benefits. However, through the survey, we saw that corn farmers are dominated in the decision-making about the selling price of the products. Approximately 74.5% households interviewed replied that corn prices are up to the buyers' valuation, about 14.3% of the surveyed people said they sold corn at the selling price according to neighbors, and 11.2% of households answered the price is based upon the agreement between the two parties. This is one of the weaknesses of the corn producing households due to limited qualifications, knowledge, and information about the market as well as poor economic accounting of the production of the households, then, the ability to negotiate when joining the chain is very limited.

Table 2. General characteristics of corn farmers in the study areas

Indicators	Unit	Muong Khuong	Simacai	Bac Ha	Average
Average age of household heads	Year	40.9	37.1	44.0	40.7
Number of household members	Person	5.1	5.7	4.6	5.1
Number of labors in each household	Person	3.2	3.2	2.9	3.1
Number of labors in each planting corn household	Person	3.1	3.1	2.8	3.0
Percentage of income from corn	%	28.4	22.9	21.7	24.3
Number of schooling year of the household heads	Grade	4.2	4.8	3.8	4.3
Experience in planting corn	Year	20.2	17.5	18.1	18.6
Agricultural acreage/household	m^2	9,690.9	8,034.5	9,247.0	8,990.8
Corn acreage/household	m^2	6,700.4	5,615.9	5,900.1	6,072.1
Number of plots for corn planting/household	slot	1.8	2.2	2	2.0
Average corn productivity	ton/ha	4.3	3.4	3.8	3.8
Corn yield/household	Tons	2.88	1.91	2.24	2.33

Source: Synthesis from surveys, 2016

Table 3. Characteristics of small collectors in the villages/communes

Indicator	UNIT	Average	Min	Max
Age of the collectors	Year	42.5	31	52
Business experience	Year	12.5	3	23
Laborers for corn collecting	Persons	1.3	1	2
Yield of corn collected annually	Ton	38	13	55
Percentage of loss	%	2.8	1	3.2
Income from corn business	Million VND/year	10.5	4.2	16.5

Source: Synthesis from surveys, 2016

Table 4. Characteristics of the district-level collectors

Indicator	Unit	Average	Min	Max
Age of the collectors	Year	44.6	32	60
Business experience	Year	13.5	5	30
Laborers for corn collecting from family	Person	1.5	1	2
Hired labor	Person	1.6	1	5
Yield of corn collected annually	Ton	250	80	800
Percentage of loss	%	3.8	2	5
Income from corn	Million VND /year	48	15	150

Source: Synthesis from surveys, 2016

3.1.3.3. Local small collectors (in villages/communes)

* Characteristics of capacity

The corn collectors in villages and communes in the three studied districts could be divided into two types of collectors, namely mobile collectors and collectors at grocery shops in the villages/communes. Collectors in the villages and communes have approximately 13 years of business experience on average. They mostly use family laborers for doing business, 1.3 people on average. The quantity of corn purchased by the collectors varies significantly, with some buying 13 tons/year and some others reaching 52 tons/year.

* Functions/activities

Mobile collectors and traders usually deliver living necessities, such as rice and food, to a farmer's house by motorbike, so these collectors get prices that are 10-15% lower than the market prices. They will sell corn to the district-level collectors or to collectors at the grocery shops. There are not many collectors following this type (2-3 people/communes), thereby the total quantity of corn purchased in this type is low. Collectors - grocery shops sell living necessities, agricultural production inputs. and seeds, and at the same time they purchase agricultural commodities, including corn. They often sell fertilizers, seeds, and essential supplies (rice, food, etc.) for corn producing households who then sell their products at the shops or at home. The shops typically purchase corn at lower prices than the market prices (by 5-10%). Nevertheless, corn producing households still sell corn to the shops because they have a strong relationship; they can help poor households in difficulty or they provide materials and seeds for corn producing households, and in exchange, households have to sell their products. The number of collectors in this type in the various commune's studies ranged from 5-7/ commune so their quantity of corn every year is rather large from 13-52 tons/ year / household.

3.1.3.4. Collectors at district-level

* Characteristics of capacity

As can be seen from Table 3.5, the district-level collectors have 13.5 years of business experience on average. The collectors have an average of 1.5 employees from their families, but corn procurement activities require a lot of male labor for portage, transportation, drying, and bagging of corn. Therefore, large collectors are more likely to hire more workers to work, and the average number of employees hired is 1.6 people/collector. Besides, during the corn purchasing season, there is a greater need to transport workers, porters, and dry corn, so they hire seasonal workers. The quantity of corn procured by these units is quite high. According to estimates, the district-level collectors gathered 250 tons / year on average, the highest of which was 800 tons / year.

* Functions (activities)

District-level collectors purchased corn from communes then sent it to primary processing and packaging, and rented trucks to sell corn to the traders in the province (the agri-products export and processing enterprises) and other provinces. A number of collectors purchased fresh corn (after splitting seeds) at a price equal to 65% of the price for dried corn, then hired laborers to dry the corn until approximately 15% humidity, bag the corn, and transport it as required by the collectors in the province. Most collectors in the province paid 50 to 70% cash back on purchases. However, the premises outside of the province often overlap the payment period or pay after 10-15 days (accounting for 30- 50%).

3.1.4. Analysis of the linkages between stakeholders in the corn value chain

In the corn value chain, the stakeholders are linked to each other, however this is just temporarily or seasonally, as shown below:

The linkage between corn producing households and input suppliers is relatively tight. For agents selling materials and seeds, they offer cash or deferred cash payments/payment on credit (about 50%) for the value of the materials but take into account the bank interest rates are higher by (1-2 %). Small collectors - grocery shops at the commune sell living necessities and purchase corn at the same time, and about 60-70% of the corn producing households buy basic necessities in the form of debt signed. The collectors also offer loans (with high interest rates) and help poor households in cases of sickness. Large district-level collectors often invest indirectly through collectors in communes (such as loans or investments in agricultural materials with low interest rates). Small collectors in the communes are considered as agents selling agricultural materials and collecting/buying corn from commune-level collectors. Then, the commune collectors sell products to district-level collectors at the agreed price. About 18-20% of households with economic and transporting capacity contact district-level collectors to sell products. The link between them and the corn farmers is not close, as the collectors only buy corn when the farmers want to sell, and without contracts at the price decided by the collectors.

Collectors in the corn value chain usually exchange information on yield, market prices, and their buying prices. A number of commune-level collectors borrow money or agricultural materials from district-level collectors, and then the district-level collectors will buy corn from these farmers. When receiving corn from commune collectors, the district-level collectors deduct the lending money or pay in cash from 70-100%. The district-level collectors contact collectors inside or outside of the province via telephone in order to make oral agreements. The relationship between the collectors in the chain is through the purchasing of products (so called partners). They often have no contracts or debentures signed by both parties.

Collectors and retailers often have long-term close business relationships. Retailers buy corn primarily from a fixed collector and usually enjoy preferential prices and times of payment. The retail agents are often located in central districts and cities (town of Bac Ha, Lao Cai City).

Table 5. Economic performance of corn production of household groups in the communes participating in the program, 2015 (average of 1 ha of corn planted land)

Indicator	UNIT	Muong Khuong	Simacai	Bac Ha	Average
Gross output (GO)	1000 VND	20,425.0	15,300.0	17,670.0	17,798.3
Intermediate costs (IC)	1000 VND	5,175.5	4,555.0	4,901.5	4,877.3
In-kind costs (family labor)	1000 VND	6,118.5	4,601.0	5,433.1	5,384.2
Total costs (TC)	1000 VND	11,293.9	9,156.1	10,334.6	10,261.5
Value added (VA= GO-IC)	1000 VND	15,249.5	10,745.0	12,768.5	12,921.0
Profit (Pr=GO-TC)	1000 VND	9,131.1	6,143.9	7,335.4	7,536.8
VA/IC	Times	2.95	2.36	2.61	2.64
Profit/TC	Times	0.81	0.67	0.71	0.73

Source: Synthesis from surveys, 2016

3.1.5. Economic analysis of the corn value chain

* Analysis economic efficiency of production of the corn farmers

An analysis of the economic performance of corn production was conducted in order to determine profits obtained by corn farmers in the study area, which will help guide farmers to make more effective investments in producing corn in the following growing period.

The results in Table 3.6 show that the total gross output from 1 ha of cross-bred corn planted on the land of surveyed households averaged 17.8 million VND/ha, of which, corn producing households in Muong Khuong achieved the highest production with approximately 20.4 million VND/ha, households in Bac Ha came next (approximately 17.67 million VND/ha), and households in Simacai had the lowest production with 15.3 million VND/ha. The reason for the gaps is due to the different corn productivities, as well as the quality of corn varying by location leading to the differences in prices of corn (in Simacai and Bac Ha, the prices of corn were lower than those in Muong Khuong).

Intermediate Costs: intermediate costs for corn production of the surveyed households averaged nearly 4.9 million VND/ha, equivalent to 47.5% of the total investment costs. By comparing the three districts, the underlying trend of the intermediate costs is the same as the trend generated for gross output. Of the intermediate costs for corn production of the households, the cost of fertilizers accounts for a large proportion of the input costs for corn production.

Family labor costs: Labor costs for corn production, including an estimation of the cost of family laborers based on the costs of hiring local employees for production and harvesting corn, are nearly 5.4 million VND/ha on average, occupying 10 - 15% of investment costs (mainly for large-scale production households during the period of seasonal stress).

The average value added calculated for 1 ha of planted corn in all households surveyed was 12.9 million VND/ha, the highest was in Muong Khuong (15.2 million VND/ha) and the lowest was in Simacai (10.7 million/ha). Similarly, the average net profit calculated over 1 ha of corn planted of farmer households was 7.5 million VND/ha, the highest was in Muong Khuong (9.1 million VND/ha) and lowest was in Simacai (approximately 6.1 million VND/ha).

* Analysis of economic performance of each stakeholder in the corn value chain

To examine the distribution of interests among stakeholders in the corn value chain, we selected the market channel 2 in corn production to conduct an economic analysis, and planning and calculation results of the economic performance of production and business in the corn value chain stakeholders are shown in table 6.

Table 6. Economic performance of production and business of the stakeholders in the corn value chain in the study areas, 2015 (accounted for 1 ton of dry corn)

Indicator	Unit	Corn farmers	Commune collectors	District-level collectors	Retailers
Channel 1: Corn farmers - Commune collectors - District-level collectors - Retailers					
Gross output (GO)	1000 VND	4,647.0	5,137.0	6,146.0	6,600.0
Intermediate costs (IC)	1000 VND	1,272.3	4,785.2	5,863.6	6,097.4
Total costs (TC)	1000 VND	2,676.9	4,839.0	5,910.8	6,270.5
Value added (VA= GO-IC)	1000 VND	3,374.7	351.8	282.4	502.6
Profit (Pr=GO-TC)	1000 VND	1,970.1	298.0	235.2	329.5
Profit/TC	Times	0.74	0.06	0.04	0.05
Channel 2: Corn farmers - District-level collectors - Retailers					
Gross output (GO)	1000 VND	5,137.0		6,146.0	6,600.0
Intermediate costs (IC)	1000 VND	1,490.2		5,863.6	6,097.4
Total costs (TC)	1000 VND	2,718.7		5,910.8	6,270.5
Value added (VA= GO-IC)	1000 VND	3,646.8		2,82.4	502.6
Profit (Pr=GO-TC)	1000 VND	2,318.3		2,35.2	329.5
Profit/TC	Times	0.86		0.04	0.05

Source: Synthesis from surveys, 2016

Table 7. Distribution of value added and net profit of the stakeholders in the corn value chain in the surveyed areas, 2015

Stakeholders	Value added (VA)		Net profit (Pr)	
	Amount of money (1000 VND/ ton)	Percentage (%)	Amount of money (1000 VND/ton)	Percentage (%)
Channel 1: Corn farmers - Commune collectors - District-level collectors - Retailers				
Corn farmers	3,374.7	74.80	1,970.1	70.4
Commune-level collectors	351.8	7.80	262.0	9.4
District-level collectors	282.4	6.26	235.2	8.4
Retailers	502.6	11.14	329.5	11.8
Total	4,511.4	100.00	2,796.8	100.0
Channel 2: Corn farmers - District-level collectors - Retailers				
Corn farmers	3,675.3	82.4	2,372.1	80.8
District-level collectors	282.4	6.3	235.2	8.0
Retailers	502.6	11.3	329.5	11.2
Total	4,460.3	100.0	2,936.8	100.0

Source: Synthesis from surveys, 2016

Channel 2: Corn farmers - District-level collectors - Retailers- Consumers

Corn farmers: Intermediate costs of corn farmers was very low at 1,490.2 thousand VND/ton of corn, and corn farmers sold products to district collectors at an average price of 5,137 VND/kg. Value added generated by corn farmers was 3,646.8 thousand VND/ton, and after deducting the costs for gasoline and transportation, the net profit for corn farmers was 2,318.3 thousand VND/ton corn, which was higher than the net profits when farmers sold to commune-level

collectors (402.03 thousand VND / 1 ton of corn). Net profit increases by 20.04% in comparison with the profits observed in market channel 1.

* Distribution of value added and net profit of the stakeholders in the corn chain

Through analyses on the distribution of value added and net profit, we can see the distribution of benefits in the value chain to the stakeholders, which will be used as the basis to evaluate the effectiveness of the distribution channels and identify the consumption channel bringing about the most benefits for the value chain, especially for the corn farmers. Results from the evaluation on the distribution of value added and net profit to the stakeholders in each market channel are shown in Table 7.

Marketing channel 2: Corn farmers - District-level collectors - Retailers- Consumers

Distribution of value added (VA): The total value added of market channel 2 reached 4,460.3 thousand VND/ton of corn, of which corn farmers gained 3,675.3 thousand VND/ton of corn (82.4%), retailers gained 502.6 thousand VND/ton of corn (11.3%), and lastly, district-level collectors gained 282.4 thousand VND/ton of corn (6.3%).

Distribution of net profit (Pr): The total net profit of the value chain was 2,936.8 thousand VND/ton of corn, of which corn producers gained 2,372.1 thousand VND/ton (80.8%), retailers gained 329.5 thousand VND/ton of corn (11.2%), and lastly, district-level collectors gained 235.2 thousand VND/ton of corn (8.0%).

The analysis on the distribution of profits to the stakeholders in market channel 2 showed that when the producers sold products directly to district-level collectors, the producers received a higher proportion of value added (increasing by 7.6%) and the net profit for the producers rose by 10.3% than when the producers sold products directly to collectors in the communes. These figures should be paid more attention to by participating organizations to aid producers in finding new markets, organizing collector groups, providing support for transportation costs, and signing contracts for consuming outputs so that they can sell products directly to the district, province collectors, animal feed processing companies, or retail agents, thus shortening the distribution channel in order to improve profits for corn producers in the study areas.

3.1.6. Strengths, weaknesses, opportunities, and threats for the corn value chain (SWOT)

The analysis of the strengths, weaknesses, opportunities, and threats will be the basis to prepare a product development strategy. Limiting the weaknesses and threats while promoting the strengths and opportunities to develop and enhance the corn value chain is the purpose of this study. The results of the SWOT analysis of the corn value chain are illustrated in Table 8.

3.2. Solutions to improve livelihoods of minority ethnic households in Lao Cai province via developing corn value chain

Studying the corn value chain in the mountainous Lao Cai province in three districts (Muong Khuong, Simacai, Bac Ha) showed that due to uneven literacy, a high ratio of poor households in some communes, limited technical proficiency, and a limited capacity in corn production and market access of farmer households, the development of an effective corn value chain will require implementing comprehensive and synchronized measures to enhance capacity, technical innovations, manufacturing support, and marketing. Therefore, we propose a number of solutions as follows:

3.2.1. Strengthening the capacity for cadres and stakeholders of the value chain

Strengthening the common interest groups, conducting training on corn production technology and production planning, marketing, conducting field trips to learn from other models in Vietnam, and training for cadres from agriculture extension centers/stations villages/communes to transfer the knowledge to farmers in their villages.

3.2.2. Finding new markets and shortening the distribution channel of corn commodities

Connecting the market (organizing workshops, trade fairs to find new markets), supporting the common interest groups to link enterprises and cooperatives to consume the products, and supporting transportation costs for the enterprises/cooperatives who buy corn from the common interest groups and farmer households.

3.2.3. Upgrading the quality of products and reducing the investment costs through post-harvest activities

Providing support in procuring seeds and fertilizers, establishing pilot models and exhibiting models for new high-yield and good quality corn varieties, and instructing common interest groups on planting, growing, and preservation of corn post-harvesting.

Combining the training on planting, growing corn technologies and training on post-harvest corn preservation technologies using knowledge appropriate for the conditions of the local households, especially the preservation technology for whole corn (because by custom, most of the households from ethnic minorities preserve the whole corn ears).

Also, we can support small-capacity corn dryers (using firewood) to improve the quality of corn, and prolonging preservation to improve the value of corn commodity. Currently, according to the corn farming practices, households still leave corn in the field and do not harvest it immediately, resulting in high moisture and decreased quality of corn, especially in the rainy season. So, if there is a small dryer for each cluster of villages / communes, it will help households improve corn preservation, and increase the quality of corn, selling price, and production efficiency.

Table 8. SWOT analysis of the corn value chain development in the study areas

Strengths (S) Large corn planted acreage Abundant labor Hardworking people with long years of experience in corn production Appropriate soil and climate conditions for growing corn, suitable for the poor invest in Easy to plant, grow, primarily process, and preserve corn crops Huge demand from the market, collectors buy corn locally	Weaknesses (W) Due to steep and fragmented corn planted land, corn cultivation is difficult and cost of production is high Low literacy, lack of knowledge about science and technology of cultivation, husbandry and growing, harvesting, and preserving corn Poor farming practices, a number of households produce corn following the method of extensive corn cultivation, leading to low-productivity, pests, and decay Lack of capital for production, buying materials on credit then paying back in products (corn), usually leading to extortion Producers have limited capacity to negotiate / bargain with the sellers Limited access to market information or lack of capacity to approach market information Very low capacity for farming and economic accounting management of the households Lack of links among the value chain stakeholders Inefficient post-harvest preservation (lack of storage areas, no dryer), leading to low quality corn Lack of water as corn is usually grown in mills without irrigation systems (depend heavily on the weather)
Opportunities (O) There are projects locally deployed creating opportunities for people to improve their knowledge in production The state support programs for mountainous districts, such as 135, and there are a number of provincial support policies to boost corn production and consumption The large potential consumption market for corn as Vietnam still has to import corn from abroad for processing animal feed A number of high-yield cross-bred corn varieties and new advanced technologies are available to apply to production	Threats (T) Increasing input prices; the prices of fertilizer and corn seed are increasing, while the quality of corn varieties, materials, and fertilizers are difficult to control Changing weather and climate; prolonged dry, hot, and drought seasons, leading to poor crops; or chilling cold and biting cold, affecting corn productivity Serious pest damage Increasingly fierce competitiveness in the market, due to the import of genetically modified corn and corn production in neighboring provinces with better quality and lower costs Unstable prices (depreciation season)

3.2.4. Strengthening advocacy activities to raise awareness of corn production farmers

Organizing dissemination campaigns through the mass media of the communes/villages (commune/village speakers), the integration of activities into the meetings of communist party at commune and village levels and other socio-politic organizations, and community meetings in order to disseminate information. Besides, group tours could be organized for the common interest groups to learn corn production models, applications of new technologies, and technological advances in the production of corn in order to improve efficiency and incomes of farmer households in this region and create good linkages with companies/enterprises in order to change the mindsets of corn farmers from the backward production practices to market-oriented production (selling fresh corn to companies when it is on the field).

4. CONCLUSIONS AND IMPLICATIONS

Hybrid corn is one of the key crops, contributing greatly to creating jobs, producing income, and ensuring food security for local populations, particularly in the mountainous communes which are home to many poor households and people from ethnic minorities in Lao Cai province.

Economic performance of corn production of households in the three districts have certain differences: for every VND invested in corn production, the households will gain about 2.64 VND of value added and the average profit/costs ratio for all of the surveyed households reached 0.73 times. Distribution of profits to stakeholders in the value chain is still not reasonable, our producers participated in planting and growing, and struggled during the 3-4 months of intensive work, but they only obtained 70.4% of the profit, while corn traders joined the business for just a short time, but they earned 29.6% of the total profit of the value profit chain.

Product distribution channels are long with many stakeholders involved. Almost 75% of the corn producing households sold their products to the traders in their communes and then resold to other stakeholders (district, province, retail) before the products arrived to the consumers. So, the profits for corn producers were lower than if they had sold directly to large district collectors. As market information is limited, people often sold corn to commune collectors and traders. Price extortion and involuntary selling to collectors in communes / villages has been happening in the mountainous communes, especially for a high proportion of ethnic minorities, and unstable prices are not good for corn producers.

Hybrid corn is used as raw materials for food processing for humans (corn products, cooking wine) and cattle. However, the corn must be transported to the lowland provinces of Hanoi, Hai Duong, and Hung Yen, and some Southern provinces such as Dong Nai and Ho Chi Minh for food processing, leading to high transportation costs. Currently, the link between corn farmers and other stakeholders is very loose, so the consumption of corn products is difficult and heavily dependent on the local traders and the China market.

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PIG PRODUCTION AND FARM INCOME IN THE PIG VALUE CHAIN IN HUNG YEN AND NGHE AN PROVINCES

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Received date: 13.07.2017 Accepted date: 14.09.2017

ABSTRACT

The livestock sector in general and pig production in particular is important in Vietnam not only for supplying food for its growing population but also for its significant contributions to the country's economy. Smallholder pig producers which are predominant, supply at least 80% of pork in the market in the country. This paper aims to draw a picture of pig production performance of small farmers based on the survey data of ILRI-VNUA using a value chain approach. It is estimated that the income of pig smallholders was, on average, VND 765 thousand per 100 kg pig live pig weigh; regional differences in income were statistically significant. Pig diseases, feed use, and measures applied for disease protection had significant effects on farm income. However, different perceptions on food safety were not significantly associated with income levels of farmers. A long time is likely to be needed to change the perception and behaviors of farmers on food safety.

Keywords: Income, pig value chain, smallhoder.

Chăn nuôi lợn và thu nhập của hộ trong chuỗi giá trị thịt lợn ở tỉnh Hưng Yên và Nghệ An

TÓM TẮT

Ngành chăn nuôi nói chung và chăn nuôi lợn nói riêng ở Việt Nam có vai trò quan trọng không chỉ cung cấp thực phẩm cho người tiêu dùng với quy mô ngày càng gia tăng, mà còn quan trọng bởi sự đóng góp đáng kể vào nền kinh tế quốc gia. Các hộ chăn nuôi nhỏ lẻ phổ biến và cung cấp khoảng 80% lượng thịt lợn cho thị trường. Bài báo này mô tả tình hình chăn nuôi lợn của các hộ chăn nuôi nhỏ tỉnh Hưng Yên và Nghệ An, sử dụng số liệu điều tra của ILRI-VNUA trong chuỗi giá trị. Kết quả cho thấy thu nhập từ chăn nuôi lợn của hộ là 765 nghìn đồng cho 100 kg thịt lợn hơi và có sự khác nhau lớn giữa 2 tỉnh. Dịch bệnh, sử dụng thức ăn, các biện pháp phòng trừ dịch bệnh có ảnh hưởng đến thu nhập từ chăn nuôi lợn. Tuy nhiên, thu nhập của các nhóm hộ có nhận thức về vệ sinh an toàn thực phẩm với thịt lợn không khác nhau. Do đó để thay đổi ý thức của người dân đối với vấn đề an toàn thực phẩm sẽ đòi hỏi thời gian dài hơi hơn.

Từ khóa: Chuỗi giá trị thịt lợn, hộ chăn nuôi, thu nhập.

1. INTRODUCTION

Livestock production contributed to about 18% of Vietnam's total GDP in 2010 and this figure is expected to rise up to 20% by 2020 (MARD, 2012). The pig sector consistently contributed about 74-80% of the total meat production in Vietnam during 2000 - 2012 (Nga *et al.,* 2014). Small-scale production predominates in the pig sector, with more than 4 million pig-raising smallholders in the country, of which, 52% are raising 1-2 pigs (GSO, 2011), and supplying at least 80% of Vietnam's pork consumption (Lapar *et al.*, 2010; Lapar and Tiongco, 2011). Income from pig production is important because it provides a

source of quick cash in times of emergency or a shortfall in household cash requirements due to a crop failure, medical emergencies, a family death, natural disasters, or other reasons. Therefore, the pig sector is critically important in agriculture and rural economies, especially to small farmers.

This paper aims to characterize the pig production and income of the sector, focusing on smallholders in the pig value chain in Northern Vietnam, and draws attention to important implications for improving income from participation in the pig value chain.

2. SITE DESCRIPTION AND METHODS

2.1. Site description

Hung Yen and Nghe An are provinces with fairly high pig herd sizes in the North of Vietnam, which are estimated to have more than one million and 623 thousand heads, respectively, in 2013 (GSO, 2014). The former is located in the Red River Delta and represents a more developed production, while the latter is located in the Northern Central Coast and represents a more rural and less developed pig value chain (ACIAR, 2012).

2.2. Sampling and data collection

A survey was conducted in 2013 and 2014 in two provinces that were representative of different pig systems in the north. The site selection was implemented as follows: In each province, a group discussion with the local governments and departments of agriculture and rural development was conducted, and as a result, three districts were chosen representing different pig systems and value chains. In Hung Yen province, Tien Lu, Van Giang, and Khoai Chau districts were selected, while in Nghe An, they were Hung Nguyen, Do Luong, and Dien Chau districts. In each district, three communes were selected randomly based on pig density groups (low, medium, and high); a total of 18 communes in 6 districts were finally picked. Farmers were then randomly chosen from the list of pig farmers provided by veterinary staff in the communes. The total sample size was 318 farmers who produced finished pigs. In addition, focus group discussions involving different value chain actors were also conducted for mapping and describing the pig value chain in the study sites.

Descriptive comparative statistics and gross margin analysis were employed to characterize farmers and provide the basis for analysis of the economic performance of pig production. In addition, a test of the means was also used to determine the differences among groups of pig producers and locations.

3. FINDINGS

3.1. Pig production performance

Farmer profile. About one-third of the respondents were male in Nghe An province, while this figure was about two-thirds in Hung Yen, reflecting the fact that as pig production becomes a relatively more important source of income for farm households, as observed in Hung Yen than in Nghe An, it has attracted more participation from male labors. On average, a typical household size was about 4 people (Table 1). Primary economic activities of a household head were animal keeping (47% of total households) and crop production (17% of total households). Animal production was the primary activity of about two-thirds of household heads in Hung Yen (Table 1).

Scale of production. The majority of farmers reared less than 30 pigs/cycle (more than 95% in the research sites). It can be seen that the pig production scale was generally higher in Hung Yen with more than half of households raising 10-30 pigs per cycle. The pig density in both provinces was fairly low (Table 1). The reason seemed to be that farmers kept some slots of their barns empty due to low pig prices at the time the survey was conducted.

Production performance. Using information from the most recent pig cycle, a farm household was estimated to raise about 13.5 pigs, on average; there was a considerable

difference in estimates of this figure between Hung Yen and Nghe An, with the herd size in Hung Yen about 50% higher than that in Nghe An (Table 2). Farmers in Hung Yen finished a pig cycle in about 146 days, with a higher level of live weight of pigs sold than that in Nghe An. On average, a pig farm household in Hung Yen produced about 1.8 tons (live weight) per cycle, almost three times more than a typical pig raising household in Nghe An. The same picture was observed for the average live pig weight in Hung Yen and Nghe An. On average, pig farmers in Hung Yen sold pigs at a 107 kg live weight while in Nghe An the number was only 61 kg. In addition, the pig productivity in Hung Yen was higher than that in Nghe An (with 4 kg per month) (Table 2). This means that farmers in Hung Yen finished pigs in a more intensive way, having a more commercialized pig production than pig farmers in Nghe An.

Table 1. Characteristics of pig farm households

Items	Hung Yen	Nghe An	Total
Respondent as male (%)	63.7	33.2	48.6
Household head as male (%)	93.9	96.6	95.2
Household head age (years)	48.3	48.2	48.3
Household head education level (%)			
Primary	5.2	1.5	3.4
Secondary and high school	90.1	89.7	89.9
Other	4.7	8.8	6.7
Household head primary activity (%)			
Crops	14.6	20.2	17.4
Animal keeping (incl. pigs)	64.2	29.8	47.1
Other	21.2	50.0	35.5
Farm household size (people)	3.6	3.7	3.6
Housing area for pig production (m^2)	77.9	29.1	60.9
Pig density (m^2/head)	5.6	4.5	5.0
Pig herd size (% of households)			
1-10 (pigs)	39.7	77.8	55.7
10-30 (pigs)	53.8	20.0	39.6
>30 (pigs)	6.5	2.2	4.7
Water system for pig production (% hh has)	51.4	9.6	36.1
Biogas for waste treatment (% hh has)	55.7	22.1	45.8

Source: Computing from survey data by ILRI - VNUA

Table 2. Pig production in the latest cycle

Items	Hung Yen (1)	Nghe An (2)	Differences (1) - (2)	Average
Pig herd size	16.4	9.5	6.9***	13.5
Time/cycle (day)	145.9	99.8	46.2*	
Monthly gaining weight (kg)	20.1	16.1	4.0***	18.4
Total output (kg)	1,776.9	586.9	1,190.0***	1275.8
Average live pig weight (kg/head)	107.0	60.8	46.23***	87.5

Source: Computing from survey data by ILRI - VNUA. The number is calculated per household
*Note: ***,*, and ns: Significance at 1%, 10% and non-significant, respectively*

Table 3. Economic performance of pig production in the latest cycle
(1000 vnd per 100kg of live pig weight)

Items	Hung Yen (1)	Nghe An (2)	Differences (1) - (2)	Average
Total revenue	4452.5	4158.1	294.4*	4327.4
	(360.6)	*(637.3)*		*(515.2)*
Variable costs	3650.2	3472.3	177.9*	3561.9
	(618.7)	*(959.6)*		*(784.9)*
Income from pigs	822.3	685.9	136.5*	765.6
	(584.1)	*(953.5)*		*(763.2)*

Source: Computing from survey data by ILRI - VNUA
*Note: number in parentheses are standard errors; *: Significance at 10%.*

Table 4. Pig income from different production systems (unit: 1000 vnd)

Items	Income per 100 kg live pig weight			Household income from pigs		
	Nghe An	Hung Yen	T-test	Nghe An	Hung Yen	T-test
Buying breeds						
- Households self-produced breeds	943.9	809.6	134.3^{ns}	1625.0	12621.3	-10996.3***
- Households bought breeds	754.1	453.2	300.9**	-163.7	9861.0	-10024.8***
T-test	189.8^{ns}	356.4***		1788.7*	2760.3^{ns}	
Type of feed used						
- Households used mixed feeds	900.3	750.8	149.5^{ns}	476.5	12458.4	-11981.9***
- Households used industrial feeds	654.5	634.5	20.1^{ns}	4020.4	11003.2	-6982.8^{ns}
T-test	245.8*	116.4^{ns}		-3543.9^{ns}	1455.2^{ns}	

Source: Computing from survey data by ILRI - VNUA
*Note: ***,**, and *: Significance at 1%, 5% and 10%, respectively. ns: non-significant;*
The means have been tested between provinces and items.

Cost and income from pig production. On average, a farm household spent about VND 3.56 million to produce 100 kg of live weight pig, earning about VND 4.3 million in revenue and 0.76 million in income. It could be seen that the outputs and incomes from pig production in Hung Yen and Nghe An were significantly different. It was observed that all indicators of costs, outputs, and income of pig production in Hung Yen were higher than those in Nghe An (Tables 2 and 3). This may suggest that the pig systems in Hung Yen are relatively larger and more specialized than those in Nghe An.

At the research sites, pig farmers used two types of piglets, namely self-produced and bought from outside. Farmers who were raising sow or produced their own piglets had much higher incomes than those who bought piglets from the market. It is likely that self-produced piglets were of better quality and cost less than purchased piglets. In terms of feed, households using mixed feeds had higher incomes per 100 kg of live pig weight but relatively lower household incomes vis-à-vis those using industrial feed. Farmers using industrial feeds had higher total household incomes from pigs (about 80% higher). These findings have interesting implications: as scale increases, pig raising households tend to shift to using industrial feed, which is likely to be driven by labor requirements, i.e., less labor is required than when using mixed feeds. However, this may also reduce feed cost efficiency, given the relatively lower income gained per kg of live

weight of output. It thus appears that smallholders are still efficient at what they do, in the absence of other more remunerative labor use, e.g., using labor intensive feeding practices that enabled them to reduce their cash requirement for producing pigs. This situation could shift or change in the near future contingent on what economic opportunities will emerge that could raise the opportunity cost of labor in Nghe An and other similar contexts in Vietnam.

3.2. Pig diseases and income

Diseases and farmer capability to respond. Results of the survey showed that more than one-fourth of piglets got sick during most pig cycles at the time of the surveys, while about 5% of growing and fattening pigs suffered this same problem. Diarrhea was the most common disease infecting pigs (more than 90% of piglets and about one-fourth of growing and fattening pigs) (*Survey data in 2013 and 2014 by ILRI - VNUA).* However, farmers reported that porcine reproductive and respiratory syndrome (PRRS or blue ears), food and mouth disease (FMD), and head edema were the most serious diseases affecting pigs (e.g., these diseases hamper growth, prolong production cycle, and lead to death in pig production). In 2012, about 2% of piglets and growing/fattening pigs died because of diseases, with diarrhea being the most common cited disease for pigs and generating the highest cost for pig production (see Figure 2).

It was observed that the income per 100 kg live pig weight was higher and statistically significantly different between farmers having dead pigs and those without dead pigs. There were no statistical differences in income per 100 kg live weight of pigs produced between households who reported having pigs with diseases and those not having pigs with diseases. However, the household income from pigs of farmers who were applying disease protection measures was much higher than those households not applying the same measures. Farmers with larger scale pig production were always looking for the best practices in finishing pigs although they reported that their pigs had diseases. Households

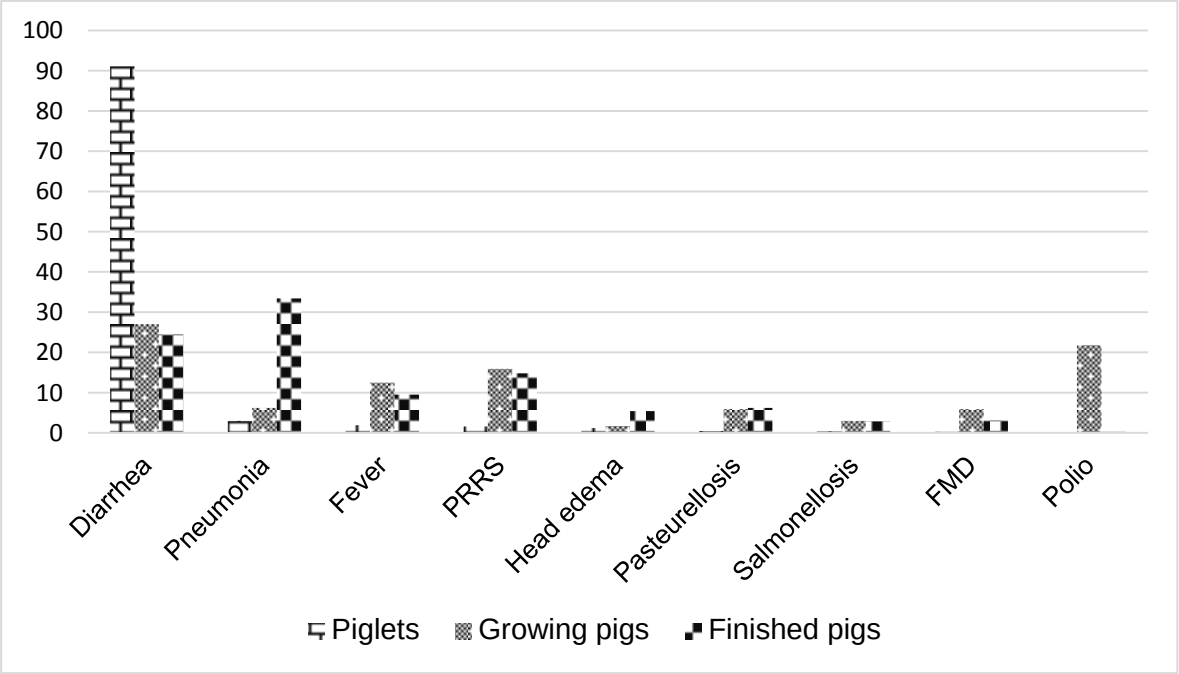

Figure 2. Disease profile in pig production *(% of pigs infected)*

Source: Computing from survey data by ILRI - VNUA

Table 5. Relationships between pig diseases and income (unit:1000 VND)

Items	Income per 100 kg live pig weight			Household income from pigs		
	Nghe An	Hung Yen	T-test	Nghe An	Hung Yen	T-test
Households having and not pig diseases						
- Having pig diseases	873.1	676.1	197.0**	1080.3	11973.9	-10893.7***
- No pig diseases	856.8	799.4	57.4ns	394.4	9373.2	-8978.8***
T-test	16.3ns	-123.3ns		685.8ns	2600.7ns	
Households having pig deaths						
- Having pig deaths	703.8	641.8	62.0ns	115.5	11144.1	-11028.6***
- Households with no pig deaths	927.3	715.5	211.8**	1205.3	11990.1	-10784.8***
T-test	-223.5ns	-73.8ns		-1,089.7ns	-846.0ns	
Measures of pig disease protection of HH						
- Disease protection measures	725.6	650.3	75.3ns	164.1	14596.4	-14432.3***
- No measures	817.3	610.5	206.8ns	347.4	7583.6	-72362.0***
T-test	-91.7ns	39.8ns		-183.3ns	7012.8*	

Source: Computing from survey data by ILRI - VNUA
*Note: ***,**, and *: Significance at 1%, 5% and 10%, respectively. ns: non-significant;*
The means have been tested between provinces and items.

with small scale production had less incentive to use disease protection measures, likely due to resource constraints. In addition, the prevalence of pig disease was likely caused by several factors. Piglet quality was limited; farmers usually bought piglets from various sources, even from open markets, with the piglet origin unclear and health status not assured. Even piglets produced in big farms were in threat of degrading in quality as reported by the department of livestock, Hung Yen province, due to the cross-breeding within pig populations over time. In addition, farming practices in pig production were not strictly regulated and compliance with prescribed breeding and hygienic standards were not strictly enforced. Vaccine utilization was not widely applied by all farmers; about two-thirds of farmers treated their sick pigs by themselves, although many of them did not feel confident in doing this. Farmers (especially in Hung Yen) reported that they lacked the knowledge and skills to diagnose and cure diseases in a timely and effective manner. About 8% of farmers sold sick pigs or slaughtered them for home consumption, and about 8% of farmers threw away dead or sick pigs. These practices might facilitate disease spread.

3.3. Income from pig production and food safety

Food safety in pork supply has become of much concern recently with widespread use of antibiotic substances and Beta-agonist, resulting in levels of residues exceeding the allowed quantities that are believed to have negative effects on human health. At present, there are no formal linkages in the pig value chains from producers to retailer, therefore, pork sold in markets could not be traced directly to their sources. Almost all farmers did not know where their pigs go to outside their districts/provinces. Similarly, most consumers did not know the origin of the pork they bought, especially from open markets in urban areas. However, consumers were willing to pay for pork with good quality (i.e., perceived to be safe, no risk to human health), with a price premium

of about 20% higher than prevailing prices. This presents an attractive market opportunity for pig farmers who could supply pork that satisfies this desired attribute.

Results of the survey showed that the income of different groups of farmers with different levels of understanding about food safety was not significantly different (Table 6). This suggests that food safety concerns still have not made inroads as a clear driver of income among pig producers, despite the apparent opportunities presented in emerging consumer demands. Future work could thus explore this in more detail, particularly in identifying clear market incentives recognized and appreciated by farmers that could drive behavior changes leading to food safety outcomes.

Table 6. Pig income and farmer perception on food safety (unit:1000 vnd)

Items	Income per 100 kg live pig weight			Household income from pigs		
	Nghe An	Hung Yen	T-test	Nghe An	Hung Yen	T-test
Understand food safety (1)	881.4	632.1	249.3**	831.5	11677.3	-10845.7***
Don't understand food safety (2)	755.3	734.2	21.1ns	1267.8	11545.9	-10277.9***
Have no ideas (3)	941.3	675.5	165.9ns	1070.8	12699.1	-11628.3***
Testing						
(1) - (2)	126.1ns	-70.6ns		-436.3 ns	2341.3ns	
(1) - (3)	-60.0ns	-38.8ns		-239.2ns	-1506.8ns	
(2) - (3)	-186.1ns	31.8ns		197.1ns	-3848.2ns	

Source: Computing from survey data by ILRI - VNUA
*Note: *** and *: Significance at 1% and 10%, respectively. ns: non-significant;*
The means have been tested between provinces and items.

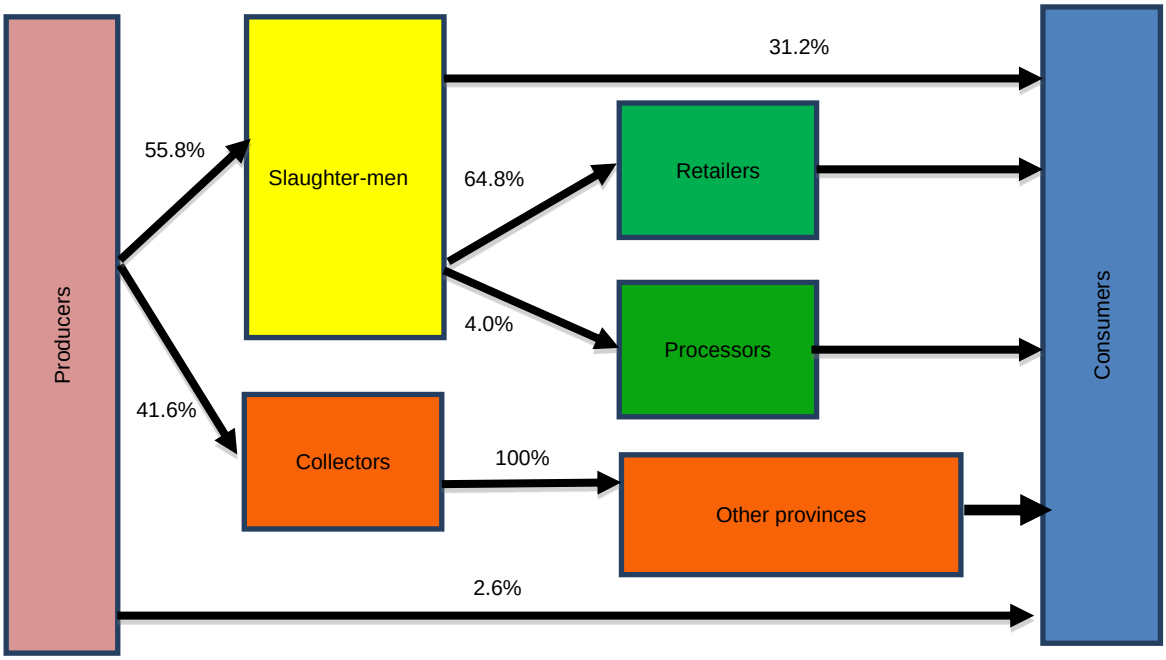

Figure 1. Map of pig value chain in Hung Yen and Nghe An

Source: survey of ILRI-VNUA and group discussion

3.4. Mapping the pig value chain

The generic pig value chain, which included input traders (feed and veterinary), pig producers, slaughters, retailers, processors, and consumers, was observed at the research sites. The longest marketing channel included all of these actors, while the shortest one had no intermediate actors between producers and consumers. Many actors performed several functions, for example, a farm household could produce pigs, buy pigs from other farmers to slaughter, process and sell raw meat, and process meat to local consumers. The results of the group discussion showed that about 60% of pigs were consumed locally (within districts and communes); only a very small part was sold directly to consumers (3% of total products) (Figure 1). More than half of finished pigs were sold to slaughterhouses (mostly slaughter men locally), and about two-fifths of produced pigs were sold to pig collectors/traders who then moved to other provinces. Few farmers (17% of total pig producers) slaughtered their pigs at home to sell directly to local consumers (in the same village) (see Figure 1). Therefore, pig traders and slaughterhouses were observed to be the most important buyers of pigs produced by smallholders in the study sites.

4. CONCLUSIONS AND IMPLICATIONS

A basic description of pig production and value chains in Hung Yen and Nghe An provinces was presented. Pig production in terms of scale, length of production time per cycle, monthly weight gain, total output, costs, and income of households were observed to vary by locations. Our estimates showed that income from pig production in Nghe An was lower than that in Hung Yen. The varying traditions of pig raising and conditions in these two provinces seemed to be the main reasons. On average, a farm household in the two provinces under study earned about VND 765 thousand for 100 kg of live pig weight. Pig diseases and their protection measures, and pig production systems (buying piglets and feeds or not) were factors affecting the income of households. Training about pig diseases and protection could be improved. The perception of farmers on food safety was limited. Farmers did not seem to have an incentive to produce safe pork. There is thus room for interventions that could lead to the improvement of farmers' understanding about animal health risks, food safety, and market demand. This remains a rich area for further research given the important implications both for peoples' well-being as well as the income and livelihoods of smallholder pig raisers.

Acknowledgements

This study was conducted under the PigRISK project "Reducing disease risks and improving food safety in smallholder pig value chains in Vietnam" (LPS/2010/047), and funded by the Australian Centre for International Agricultural Research (ACIAR) and the Consultative Group on International Agricultural Research (CGIAR), Research Program Agriculture for Nutrition and Health (A4NH). We also acknowledge the contributions to this work by the team members of the Project LPS/2010/047. We thank two anonymous reviewers and the Editor Board for their helpful comments.

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EVALUATING THE IMPACT OF VILLAGE SAVINGS AND LOANS MODEL ON LIVING CONDITIONS OF RURAL WOMEN: A CASE STUDY IN QUANG BINH AND QUANG TRI PROVINCE

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Received date: 30.03.2017 Accepted date: 02.08.2017

ABSTRACT

The study focused on the impact of the village savings and loan (VSL) model on living conditions of rural women as well as on the extension of the model, thereby proposing model development in the local conditions. The study used data from 453 respondents including member and non-member of village savings and loan models in five districts of Quang Binh and Quang Tri provinces. Site observational methods combined with difference in difference (experiment methods) methods were the main methods used in the study. Research results show that the VSL model's unique impact on gender equity, in particular, is that women can move freely without a male companion (almost 90% respondent), the impact of the VSL model increased by 6%. In addition, members in the VSL model had a poverty rate of 27%, lower than the control group of 34%. The VSL model is able to replicate to other localities in the country. The Women's Union should enhance its role in communicating model replication through participatory study tours to model regions.

Keywords: Microfinance, Quang Binh, Quang Tri, rural women, village saving loans.

Đánh giá tác động của mô hình tiết kiệm và vay vốn thôn bản đến điều kiện sống của phụ nữ nông thôn: trường hợp nghiên cứu tại tỉnh Quảng Bình và Quảng Trị

TÓM TẮT

Nghiên cứu tập trung phân tích tác động của mô hình tiết kiệm và vay vốn thôn bản đến các điều kiện sống của phụ nữ nông thôn cũng như đánh giá khả năng nhân rộng của mô hình từ đó đưa ra các đề xuất phát triển mô hình tại các địa phương có điều kiện phù hợp. Nghiên cứu sử dụng số liệu điều tra 453 thành viên và nhóm đối chứng không phải thành viên của các mô hình tiết kiệm và vay vốn thôn bản tại 5 huyện của tỉnh Quảng Bình và Quảng Trị. Các phương pháp quan sát kết hợp với phương pháp đánh giá tác động khác biệt trong khác biệt là các phương pháp chính sử dụng trong nghiên cứu. Kết quả nghiên cứu cho thấy, tác động riêng rẽ của mô hình đến nhận thức về bình đẳng giới, cụ thể phụ nữ nông thôn có thể di chuyển tự do ra khỏi nhà mà không cần một người bạn đồng hành nam, gần 90% số quan sát đồng ý với quan điểm này, tác động của mô hình tăng 6%. Bên cạnh đó, nhóm thành viên tham gia mô hình có tỷ lệ nghèo 27% thấp hơn so với nhóm đối chứng là 34%. Mô hình có khả năng nhân rộng ra các địa phương khác trong cả nước. Hội phụ nữ cần nâng cao vai trò trong truyền thông nhân rộng mô hình bằng các cuộc tham quan học hỏi của các hội viên với các vùng có mô hình.

Từ khóa: Phụ nữ nông thôn, Quảng Bình, Quảng Trị, tiết kiệm thôn bản, tín dụng vi mô.

1. INTRODUCTION

The 2008 Vietnam Microfinance Industry Assessment by the ILO argues that “for most microfinance clients, access to credit is no longer as much of an issue as is a loan that properly suits their needs and that the challenge for successful microfinance in Vietnam is thus the provision of services to poor households in more remote areas”(DIAZ,

Lillian, & Hansen, 2008). The report further argues that the demand for savings and insurance services by poor households is very high and largely unmet.

A viability study conducted by Plan Vietnam in September 2009 reveals that most households – even very poor, ethnic minority households – have access to subsidized, long-term (from 3 to 5-year) credit through the state-owned social policy banks. However, there is an acute lack of short-term credit in remote areas of Vietnam and rural households routinely borrow in informal markets at rates above 100% per annum (Plan international, 2016), usually through the purchase of food and fertilizer on shop credit.

Most poor rural households in Vietnam do not have access to savings or insurance services. The lack of a safe, transparent savings mechanism is a severe impediment to manage household cash-flows, build households' resilience to economic shocks and the right to a good quality of life. Furthermore, the combination of relatively large, long-term loan liabilities without an adequate savings mechanism or common practice of saving to service these obligations has left rural households over-indebted and acutely vulnerable.

There is a strong demand for basic financial services – particularly savings, flexible short-term credit and basic insurance services – in remote areas of the country that would enable poor households to improve their financial management and economic security. Currently, a high proportion of target households have large, outstanding long-term loans with the state-owned social policy banks. These households do not have a regular income stream or savings to service these long-term debt obligations. Families can borrow small amounts from their neighbors, but there is no formal mechanism to enable families in remote villages to access small loans.

Community-managed microfinance methodologies are recognized as the most viable mechanism to deliver financial services in remote areas; and village saving loans (VSL) methodology has proven to be an appropriate, popular and useful mechanism for poor households to save in a variety of geographic and socio-economic settings, including Quang Tri, Quang Binh province. The main objective of this study is to assess the impact of VSL model on living conditions of rural women in Quang Binh and Quang Tri provinces, and to evaluate scalability of VSL model, then to appropriate recommendation for developing VSL in new areas.

2. RESEARCH METHODS

2.1. Village savings and loan model

The village savings and loan (VSL) model was established based entirely upon community participation and action. It was implemented in close collaboration with the Vietnam Women's Union (WU) at local level. Groups of 10-25 people saved money together and took small loans from those savings. These groups were provided trainings and supports by Plan International and the WU. The activities of the VSL model run in one year cycle based on agreement of all group members and the accumulated savings and the loan profits were shared among the members according to their saving amount. The key features of VSL model were: (1) It is "savings-led" community-based with loan capital coming from the accumulated savings of members and retained earnings without external funds, (2) It is completely self-managed while Plan International and the Women's Union provide and support and monitor function; making íts own rules and managing their own money, (3) Its operations, decisions and financial procedures (savings, small-scale insurance, lending, reimbursement, imposition of penalties etc.) were done in the presence of the members without written records, (4) There is flexibility in determining the size and terms of savings and loans (including interest rates) determined by members, and (5) All savings and interest on loans are retained within the groups and no funds leave to the community. VSL model liquidates their assets and makes a pay-out to its members proportional to their savings investment at regular (usually annual) intervals.

The VSL model consisted communes that involved in VSL model from 2012 to 2015 as experimental group and communes with social economic conditions similar to experimental group in 2012 as control group. The communes that established VSL model from 2012 were designated as Roll out 1 and those communes that established VSL model from 2014 were designated as Roll out 2.

2.2. Data collection

Secondary data: Data on basic living standard, financial services from statistics of the districts and province were collected for the study. We also used data from studies which related to micro finance for rural area from various sources.

Primary data: Primary data was collected through direct interviews, Key informant interviews (KIIs), Focus group discussions (FGDs), site observations in Quang Binh, Quang Tri province (Huong Hoa district, Quang Ninh district, Minh Hoa district, Le Thuy district, this is four district which have largest number of VSL model).

Quantitative survey: In this study, the control and experiment communes were selected in the same social economic contexts of the baseline study. 280 respondents in experiment communes and 153 respondents in control communes were selected for interview.

Focus group discussions (FGDs). We conducted 9 focus groups (3 in each Rollout Group and 3 in the Comparison commune) with 8-10 participants in each. Roll out 1 and roll out 2 are different in term of time involve in village savings and loan model (Roll out 1, starting time in 2012, Roll out 2 starting time in 2013).

In this research, we used this tool to collect primary qualitative information at commune levels. It stimulated rich responses and also provides a valuable opportunity to gain insights into behaviors, attitudes, and feelings.

Key informant interviews (KIIs): Key informant interview was used in this study to develop and in-depth understanding of qualitative issues. 22 respondents including communal and village leaders and head of village and communal women unions were interviewed.

Site observation: In this study, the researchers were spot checks of any equipment, infrastructure that likely impact the involvement of the beneficiaries into the model, and collect existing/new photos on models results.

Data were gathered from a total of 433 girls/young women in 9 communes (6 communes for experiment group including 3 communes for roll out 1, 3 for roll out 2) and 3 communes of the control group). The summary of basic demographic information on girls/young women is presented in Table 1.

Table 1. Biographical information

	Unit	Experiment	Control
Number of observation		280	153
Age of respondent	years	23.1	21.1
Respondents having birth registered	%	92.8	74.2
Percent enrolled in School	%	80.4	77.8
Household size	person	4.8	4.99
Percent with children	%	77.48	58.8
Percent married	%	83.1	62.7
Average length of marriage	years	5.2	4.3
Average age of husband	years	28	27

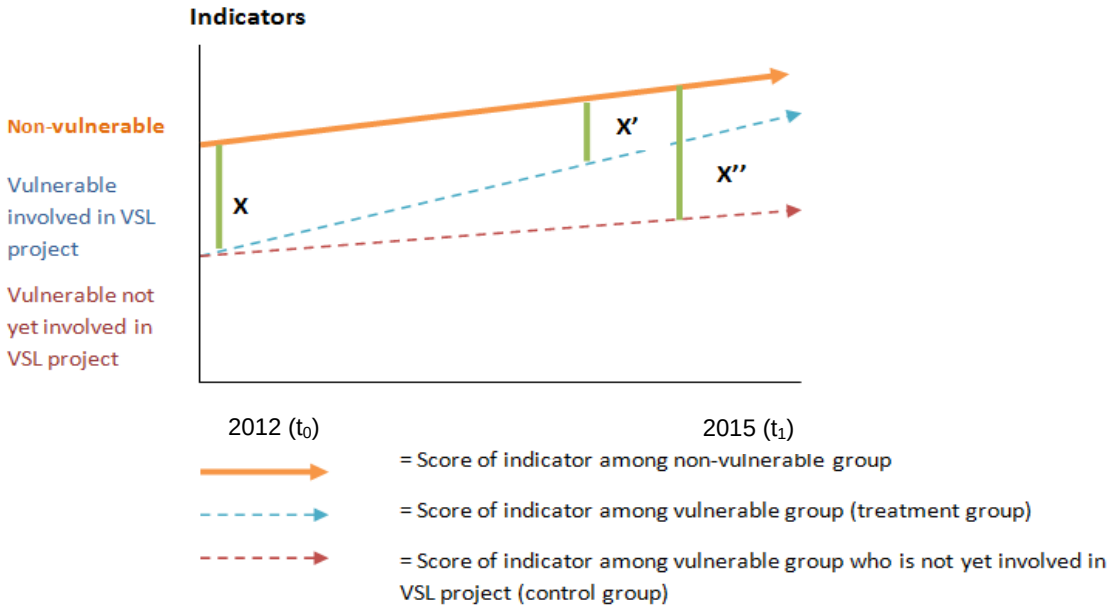

Figure 1. Hypothetical example for the advantages of using the control group in the quasi-experimental method

2.3. Data analysis

Experimental method and participatory approach were employed to analyze the impacts of the model on the living condition of vulnerable (poor, rural and ethnic minority) and non-vulnerable population (Figure 1). In this study "experimental method" was used to assess the causal impact of a program and a comparison group (control group) and also are identified and studied alongside the experiment group. The control groups represent what the intervention group would have been like if there was no model/intervention. Also, instead of only comparing before – after for interventional group, the difference between the experiment and control groups before and after program intervention is compared by using the difference-in-difference (DD) estimator method. This allows us to analyze what happen to the targeted group if the intervention had not happened. Accordingly, the evaluation is based on the change in the gap ("X") is a number of living and economic condition indicators between vulnerable and non-vulnerable households involved in the VSL model compared to the gap that existed in 2012. In other words, the comparison answers two questions:

- Has the vulnerable group (experiment group) reduced the gap with the non-vulnerable group compared to the baseline (2012)? (Compare the difference between X' and X)

- Has the vulnerable group (experiment group) reduced the gap with the non-vulnerable group because of the VSL model? (Compare the difference between X' and X")

The participatory approach was used to enhance the active participation of a wide range of stakeholders including women union, local leaders, community members, members and non-members of the VSL groups, children, etc. with special attention paid to women and girls. Both questionnaire and interview (including KII and FGD) guidelines were designed with adequate attention made on gender aspect. Gender assessment was integrated into both quantitative and qualitative data collection and analysis processes.

In this study we focus on impact of VSL model on rural women in term of the ways of spending and saving money, increasing their access to savings and loans and their households' economic status, and improving people's awareness on gender equality, intra-family relationship and the women's social status.

3. RESULTS AND DISCUSSIONS

3.1. Impact of Village savings and loan model on rural women

Information collected from the baseline survey in the year 1 and the end-line survey showed that the model brought positive impacts on VSL groups' individual members as well as on the households, communities and the WUs generally. The benefits and impacts of the model included improving beneficiaries' ways of spending and saving money, increasing their access to savings and loans and their households' economic status, and improving people's awareness of gender equality, intra-family relationship and the women's social status. Other impacts were the improvement in management skills and the ability to engage others in development activities of WU staff and members and the improvement in community relations. The model used participatory approach, which enabled women to raise their voice in their groups, community and households.

3.2. Impact of Village savings and loan model on households 'economic status and living standards

The overall target for the model is "to improve the economic security of 11,000 poor and vulnerable people in Quang Binh and Quang Tri provinces". According to Plan International's report, at the end of the model, the total number of beneficiaries including direct beneficiaries (VSL members) and indirect ones (VSL members' family members) was 35,233 people, of whom the number of VSL members was 9,865 and the number of children (both boys and girls under 16 years old) reached by the model was 12,580.

The first expected outcome of this model was to increase household income. Although the survey results did not include the household income but the positive changes in their households' economic status and living standards were shown by other indicators.

Plan International data showed that 1,932 households have got out of poverty since they joined the model. The fast growth in savings in some VSL groups was an evidence for economic improvement, as now some groups have increased the share value per member to 50,000VND and even 100,000VND (Box 1). Another indicator could be used for the poverty reduction is the percentage of people with food insecurity. Fewer people said that they are living with food insecurity after the model conducted (Fig. 2).

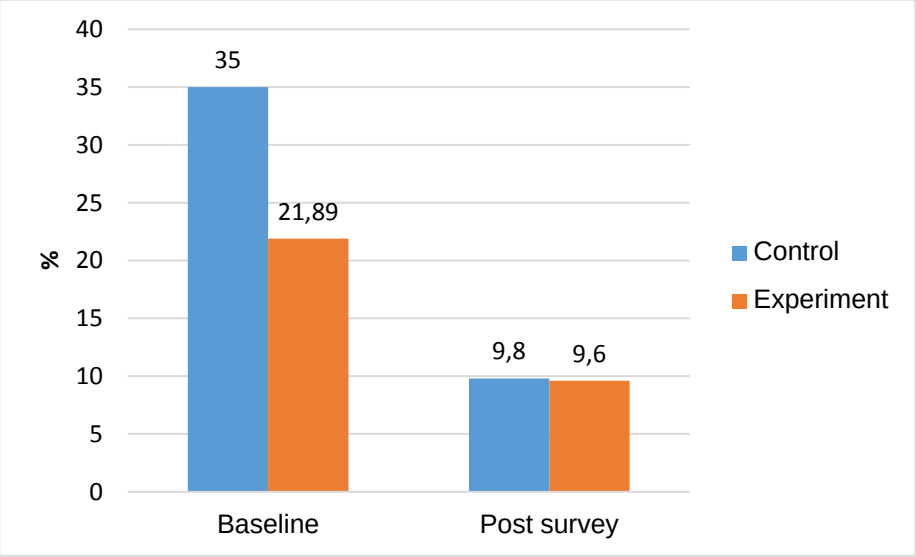

Figure 2. Proportion of people with food insecurity

Source: Synthesis from surveys, 2016

Box 1. WU staff and VSL member's opinion about impact of VSL model

"At the beginning of the model, in our group, each stamp valued 20,000VND and now it is 100,000VND. Everybody is very happy with this improvement".

"Before joining this group, my household barely had any saving as we always spent all our earning, but now, my husband and I have a saving amount of 500,000 VND to 600,000 VND every month. Since joining the group, we have bought more goods for our house such as electric rice cooker, television, and clothes for our children at the end of the year".

By joining VSL groups, members could borrow money for production and business investment, which created a first step for households to develop their economic status. Plan International data showed that around 20% of loans was used for raising livestock and 15% of that for farming. People preferred borrowing money from the groups than from other financial service providers as the loan application procedure is simple and the interest rate of VLS groups is affordable. With the same investment and production time, the low interest rate helped VSL members gain more profit.

Quote from VSL members:

"My household borrowed 3 million VND for buying piglets last year and we have just sold the grown pigs for 14 million VND. We sold 6 pigs and keep one sow for breeding. We paid all the loan and interest. Before joining the group, we had to borrow money from private money lenders with high interest but now we could easily borrow money with low interest for buying piglets and we could gain higher profit and have more money for our future investment."

"Last year, my brother gave me 5 million VND, but this amount was not enough for me to open a grocery store. Then I borrowed 2 million VND from VSL group in order to open a grocery store. After 4 months, I paid principal and interest that I had gained from the grocery store." Ho Thi Nhoang, Cu Dun village, Huong Loc commune, Huong Hoa district, Quang Tri province.

3.3. Impact of Village savings and loan model on level of awareness on financial literacy and gender equality

a. Level of financial literacy

Both quantitative and qualitative results indicated the improvement in women's financial management in terms of changing their manner of spending and saving money (Table 2, Box 2). Comparison between the baseline and the post survey results illustrated that more women knew how to save money and have their own savings. Specifically, in the baseline survey, only 10.3% of respondents in roll-out 1 and 7% in roll-out 2 said they had savings whilst the figure of roll-out 1 and roll-out 2 in the post survey was 87.3% and 79.2%, respectively (Fig. 3). In addition, in-depth interviews and FGDs information showed that women realized the necessity and usefulness of savings, hence, they did not spend money on unnecessary things but for savings (Table 2).

There has been a significant change in opinion about sources of loan. At the beginning of the model, only 30.5% and 18.6% of respondents in roll-out 1 and roll-out 2, respectively said that they had source(s) of loans but in the post survey, more than 94% of respondents in both roll-outs said they had source(s) of loans. This number in control group also increased after years but the change was smaller, from 15.2% to 54.2%. Notably, a majority of respondents in both roll-outs considered VSL/VSALs as a source of loans in urgent situation (96.45% and 91.13% respectively).

Table 2. Change in percentage of people have savings (Unit: %)

	Experiment	Control	Difference
Post survey	83.60	21.60	62.00
Baseline	8.90	1.80	7.10
Change	74.70	19.80	54.90

Source: Synthesis from surveys, 2016

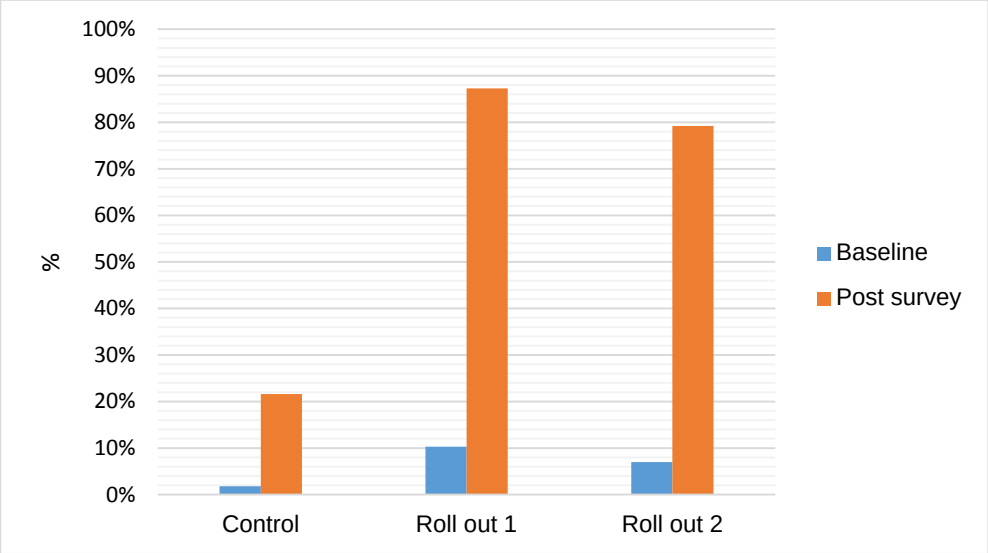

Figure 3. Household with savings

b. Level of awareness on gender equality

The end-line survey results showed that both women and men have increased their awareness on gender equality. For an illustration, the change in women's roles in making decision in households could be used as a good indicator. According to the findings of the baseline survey, "a majority of girls/young women surveyed held the view that gender equity should exist in a variety of arenas" (Evalu, 2012, p. 24), however, they did not have rights on making decision in terms of both economic and social issues. At the beginning of the model, only 16.9% and 4.5% of wemen in roll-out 1 and roll-out 2, respectively, could jointly make decision in economic development with their husbands. However, at the end of the model implementation, women's role has dramatically increased to 53.3% and 53.1%, respectively. Similarly, in making decision regarding visiting family or relatives in the post survey, 50% of interviewees in roll-out 1 and 60.8% of that in roll-out 2 said that they make it jointly with their husbands, whereas in the baseline survey the figures were only 19.5% and 5% for roll-out 1 and roll-out 2 respectively. Although the control groups experienced similar change pattern, the change was much smaller in comparison with that of experimental groups (Figures 4 and 5).

However, when being asked about who can make better decisions, they were not confident enough to say women or men; most of them refused to answer or gave an unclear answer.

"One hand alone cannot make a clap sound", hence, in order to improve the social status of women, it is critical to change the gender equality awareness of both men and women. The change in gender equality awareness also leads to the change in intra-family relationship and women's social and economic status.

3.4. Impact of village savings and loan model on intra-family relationship and women's social and economic status

As shown in Figure 5 and 6, more women jointly made decision with their husbands. This indicated that they have their voice and rights in the household affairs. Information collected from qualitative survey was consistent with findings from quantitative one. In particular, referring to the culture of Van Kieu ethnic, men are conventionally money keepers in the household although women are main labor force, but now, women could keep money and make decision on how the money be spent.

Moreover, men also joined the women in the upland fields work and helped women in doing housework (Box 4), thus, the women had more time for taking care of themselves and of the children and participating in community activities (i.e. joining WU and VSL group meetings). With regards to women's position in the community, as women had more chances to attend group meeting as well as collective activities and they became more confident to share their opinion about social issues and to participate actively in community activities. Another effect brought about by the model was that by attending VSL group meeting, women now have a place for mutual communication and information sharing. When being asked if they like to participate in community meeting (i.e. WU meetings, VSL group meetings) they all said they are happy and really like attending those meetings.

However, the changes in decision making seemed to be more significant in the households than in the community. As in community meetings, who go to and attend the meetings would be the one making decisions. The head of the household normally is the one going to attend community meeting, who is man in most cases.

Box 2. Impact of VSL model on members about level of financial literacy

"Before joining the VSL groups, women only worked during cropping and harvesting periods but now even between those periods, they also find jobs to earn money for savings"

"Before model established, I did not know how to save money, my children usually asked for 1,000 - 2,000 VND per day to buy snacks so I did not have any savings. Since I participated in VSL groups, I could save an amount of money at the end of the year and I could borrow money from VSL at difficult times." Ho Thi Nhieng (Pi Thuong), Cu Dun village, Huong Loc commune, Huong Hoa district, Quang Tri provice.

"I borrowed 1 million VND from VSL last year. I used this loan for breeding animal" Ho Thi Lan, Cu Dun village, Huong Loc commune, Huong Hoa district, Quang Tri provice.

Box 3. Impact of VSL model on member about the level of awareness on gender equality

"I do not know who can make better decisions because we always discuss with each other before deciding anything. But I think we can both do a good job" Ho Thi Lan, Cu Dun village, Huong Loc commune, Huong Hoa district, Quang Tri provice.

"I don't know. Maybe my husband will make a better decision if it is related to large purchase but for daily or small purchases, I will make decision" Ho Thi Nhoang, Cu Dun village, Huong Loc commune, Huong Hoa district, Quang Tri province.

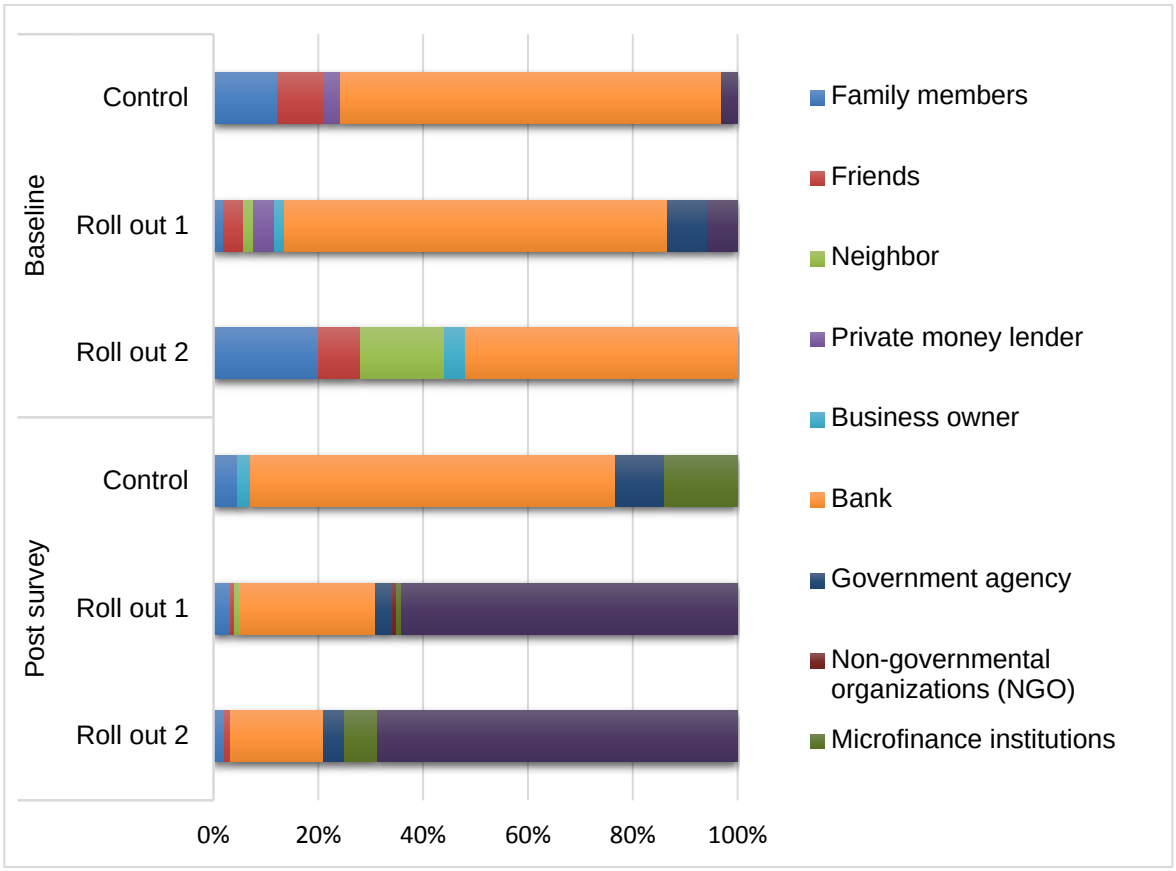

Figure 4. Source of households 'loans from last twelve month

Source: Synthesis from surveys, 2016

3.5. Impact of Village savings and loan model on management and mobilization skills of WU staff and members

One of the main model activities was to organize training courses for WU staff, Village Agents, chair and secretary of VSL groups on facilitation skills and financial and group management. The contents of these training course helped them know how to manage the savings and loans of the VSL groups, how to organize a group meeting, and how to mobilize people to join the groups and meetings, etc. These skills of WU staff and Village Agents have been not only applied for the VSL model but also for other activities of WU. Moreover, many other programs of WU have been mainstreamed into the VSL group meeting content such as training on reproductive healthcare, children healthcare, gender equality, etc. This also improved WU staff and members' propaganda techniques and ability to mobilize other people. By mobilization, WU staff and members have step-by-step changed the community, especially the men's awareness and behaviours.

3.6. Impact of Village savings and loan model on community relations

Besides impacts on individuals, the VSL model has also brought about impact on changing the community relations. First, as VSL group meeting and activities have created more chances for local people, especially women to communicate with others. They have become closer and more united. Second, besides the savings, each group had their social fund contributed by its members which is used to support the group's members to cope with emergency situation. Difficulties now can be shared with the whole community and people are brought closer together.

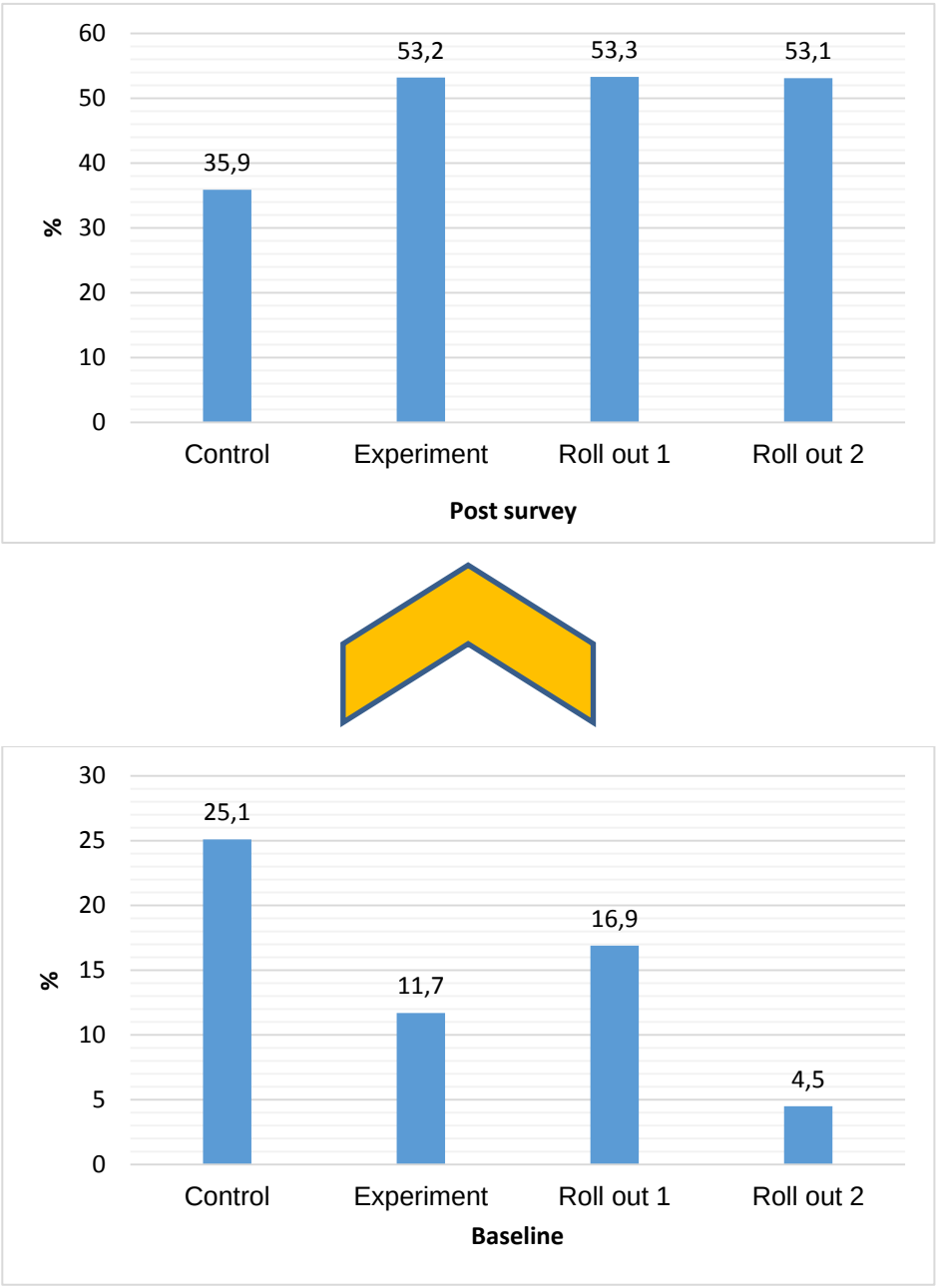

Figure 5. Change in making large household purchase decisions

Box 4. Impact of VSL model on member about enhancing Intra-family relationship and women's social and economic status

"Due to Van Kieu ethnic's culture, women used to go to work in the fields and their husbands would go to markets to sell the products and kept all the money. But since the women joined VSL groups, they have known how to manage the household's finance, they have had savings and their husbands have seen the benefits of the groups as well as their wives' roles, they have changed their awareness and behaviors. Some men now go to work in the fields with their wives, let their wives keep money and even men take care of the children and housework for wives to attend the VSL group meetings".

Box 5. Impact of VSL model on WU staff and members about Management and mobilization skills

"I am happy to join the VSL group as I have more time to rest and if I attend VSL meetings I do not have to do house work at that time. At the VSL meetings, we were chatting with each other and learning about the family circumstances of each other." Ho Thi Choan, Hương Loc commune, Huong Hoa district, Quang Tri province

"Before joining this group, my household barely had any saving as we always spent all our earning, but now, my husband and I have a saving amount of 500,000 VND to 600,000 VND every month. Since joining the group, we have bought more goods for our house such as electric rice cooker, television, and clothes for our children at the end of the year".

Table 3. Change in percentage of respondent's perception about gender equity: Women should be able to move freely outside of the house without requiring a male companion (Unit: %)

	Experiment	Control	Difference
Post survey	86.40	73.9	12.50
Baseline	77.50	71.0	6.5
Change	8.90	2.90	6.00

Source: Synthesis from surveys, 2016

The impacts on women and their families have affected the girls. In this part, we would like to make it clearer about the impacts on girls in terms of awareness changes, living standards and experiment from family and community.

According to Plan International's record, the number of girls under 16 years old reached by the model was 6,493 people. These girls now had better living standards as evidenced by the decreased number of people with food insecurity. As their parents now have savings, which they had never had before joining the VSL groups, they have access to better living conditions.

Moreover, the children had more chances to go to schools and hade better access to healthcare services, as their mothers could borrow money from the VSL groups to pay for their tuition fee and health examination fee. For example, before joining the VSL groups, some women kept their children at home and never took them to the hospitals even when their children got sick as they did not have money for emergency but now they could ask for loans in short time from the VSL group with simple procedure and their children could visit doctor. Furthermore, as the mothers were provided training on children healthcare and reproductive healthcare in VSL group meetings as WU's integrated programs, the children could receive better care from their mothers. Remarkably, adolescent girls said their families are happier now as they have savings and their mothers could buy more goods for them as well as for the households.

On the other hand, not only women but also the adolescent girls showed that they basically knew about finance, loans and savings. When asked about these concepts, most of them could give interviewers basic answers such as savings means not spending money on unnecessary things, saving money in saving pigs, borrowed money means interest-free whilst loan requires payment with interest. They were taught by both parents and social communication.

In terms of experiment between boys and girls, according to both women and girls'

responses, boys and girls in the households are treated similarly in terms of attending schools. Both boys and girls have opportunities to go to school and they are treated equally at schoold. However, in Vietnam's culture, the girls normally do more houseworks than boys and this applies also for men and women. The responses for questions related to houseworks are slightly different between Kinh and Van Kieu people. As most of Van Kieu adolescent girls had dropped their schools, they had to work more than Kinh girls and they had to do both unpaid (houseworks) and paid works compared to boys who only do farming.

The response from adolescent girls also showed that there were changes in making decision right in their households. Most of the girls said that their parents made decision jointly in both economic and social decisions. But if they need money, the one they ask for will be their mother in most cases.

However, as most of the interviewed girls were quite young and did not keep money in their house, they do normally not discuss financial issues with their parents. They will ask their parents for money when they need to buy new personal belongings, to pay school fee and buy schooling tools such as books, pens, etc."

3.7. Scaling-up and recommendation

As described earlier, Plan International had piloted VSL model in Dakrong district, Quang Tri provinces where Plan International has Pus. Now the VSL model has expanded into 5 districts in Quang Tri and Quang Binh provinces (and projected to expand the number of communes in provinces where it is currently working). In addition, VSL model continues to be established in the North of Viet Nam such as Ha Giang province and Kon Tum province. The VSL model with simple characteristics, proves to be suitable with not only the ethnic minorities in Huong Hoa district, Quang Tri province and Minh Hoa district, Quang Binh province but also with the Kinh in Quang Ninh district, Quang Binh province. The VSL model is also appropriate with social economic conditions in mountainous area (Huong Hoa district) and delta area (Quang Ninh district). Both poor people and non-poor people are happy joining VSL groups. The simplicity of operational mechanism, the voluntary participation and flexible shared contribution by members can be identified as main reasons why the VSL model can be easily applied in different areas in Vietnam. According to Mrs. Thuy, Vice president of Quang Binh women union, "*A simplified model of VSL is suitable to many places, from urban to rural areas, and from Kinh people to ethnic minorities*".

Based on the positive impact of VSL model, in the next period the Women Union and Plan International should maintain and repeat training courses on group management skills for VSL group leaders and secretary. Although the paperwork is simple, with low education level and language barriers, the group leaders and secretaries still encounter difficulties in remembering all the financial management techniques. Moreover, they have limited capacity to train other people on these skills when they have a switch in the leadership and secretary positions.

Recommendation is also made for promoting the benefits of the VSL model in order to replicate to other locations. WU should organize more study tours for members to visit successful VSL models supported by the WU in targeted model areas.

4. CONCLUSION

The VSL model has brought positive impacts on VSL groups' individual members as well as on their households, community and the WUs generally. The benefits and impacts of the model include the followings: improving beneficiaries' ways of spending and saving money, increasing their access to savings and loan and their households' economic status, and improving people's awareness on gender equality, intra-family relationship and the women's social status. Other impacts which were raised during the survey were the

improvement in management skills and the ability to engage others in development activities of WU staff and members and the improvement in community relations. The model used participatory approach, which enabled women to raise their voice in their groups, community and households.

The overall target for the model is "to improve the economic security 11,000 poor and vulnerable people in Quang Binh and Quang Tri provinces". According to Plan's report, at the end of the model, the total number of beneficiaries including direct beneficiaries (VSL members) and indirect ones (VSL members' family members) attains 35,233 people, of whom the number of VSL members is 9,865 and the number of children (both boys and girls under 16 years old) reached by the model is 12,580.

Both quantitative and qualitative data indicated that the improvement in women's financial literacy in terms of changing manners in spending and saving money. There is a significant change in respondents' opinion about sources of loans. At the beginning of the model, only 30.5% of respondents in roll-out 1 and 18.6 of that in roll-out 2 said that they had source(s) of loans but in the post survey, more than 94% of respondents in both roll-outs said they can have access to source(s) of loans. This number in control group also increased after years but the change was smaller, only from 15.2% to 54.2%. Noticeably, a majority of respondent in both roll-out 1 and roll-out 2 considered VSL/VSALs a source of loans in urgent situation (96.45% and 91.13% respectively). Moreover, not only women but also the adolescent girls revealed that they have basic knowledge about finance, loan and savings.

If the baseline survey showed that only women had good awareness of gender equality, the data from the end-line survey shows that both women and men have increased their awareness of gender equality. As the gender awareness of men has increased, women could raise their voice in the households as well as in the community and their social and economic status has both improved.

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