
Effective of *Neosartorya* and *Talaromyces* to control *Alternaria brassicicola* causing leaf spot of kale

Punyanobpharat, A.¹, Soyong, K.² and Poeaim, S.^{1*}

¹Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Ladkrabang, Bangkok, Thailand; ²Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Ladkrabang, Bangkok, Thailand.

Punyanobpharat, A., Soyong, K. and Poeaim, S. (2018). Effective of *Neosartorya* and *Talaromyces* to control *Alternaria brassicicola* causing leaf spot of kale. International Journal of Agricultural Technology 14(7): 1709-1718.

Abstract The antagonistic potential of *Neosartorya* and *Talaromyces* proved to control leaf spot of kale caused by *Alternaria brassicicola*. Leaf spot symptoms were collected from Nakornpathom, Suphanburi, Ratchaburi and Nonthaburi provinces, isolated and identified the pathogen by morphological and molecular characterization. Four isolates were confirmed as *A. brassicicola*. The pathogenicity test was done by detached leaf bioassay and found that AbK-NP01 was the highest virulence pathogenic isolate. Dual culture technique was used to assay with eight species of antagonistic fungi (*N. hiratsukae*, *N. pseudofischeri*, *N. aureola*, *N. spinosa*, *N. fennelliae*, *Neosartorya* sp., *T. trachyspermus* and *T. muroii*) against *A. brassicicola*. The best effective of antagonist to control the tested pathogen was *N. spinosa* CHA09-A01.

Keywords: Leaf spot disease, *Alternaria brassicicola*, *Neosartorya*, *Talaromyces*

Introduction

Kale (*Brassica oleracea*) belongs to *Brassicaceae*. The native growth of kale is Asia. In Thailand, kale is cultivation around 122 million square meter and total productivity at 102,405 tons/year (Department of Agriculture Extension of Thailand, 2015). From this result make kale be important economic crop but large problems are plant disease which able to damage, loss and cost effect for agriculture. Disease of plant in genus kale such as damping-off cause by *Pythium* sp., *Fusarium* sp., *Rhizoctonia solani* (Pscheidt and OCamb, 2018) and leaf spot cause by *Alternaria brassicicola* (Rimmer *et al.*, 2007, Iacomini *et al.*, 2004).

This study interesting in leaf spot disease caused by *A. brassicicola* because of can loss productivity at 40% especially in rainy season (Anan and

* Corresponding Author: Poeaim, S.; Email: poeaim@hotmail.com

Athinuwat, 2016). The data record from Bureau of Epidemiology of Thailand (Siripanich, 2013) found that the most farmers are use chemical to control disease and had been affect to farmer, consumer and environment. Including record from 2003-2012 of patient 17,340 persons which average about 1,734 persons/ year and patient rate 2.35/hundred-thousand persons. So, we would like to develop biological control to control plant disease alternative synthetic chemicals. By choosing to study effective of *Neosartorya* and *Talaromyces* species as antagonists to control leaf spot disease from *A. brassicicola*. Which Boonsang *et al.* (2014) have been presented ethyl acetate extracts of *Neosartorya* sp. against ten plant pathogenic fungi and Dethoup *et al.* (2018) have been used *T. tratensis* to against rice brown spot and dirty panicle.

The objective of this study was to evaluate *Neosartorya* and *Talaromyces* species to control leaf spot of kale caused by *A. brassicicola*.

Material and methods

Isolation of pathogen

The pathogen were isolated from leaf spot symptoms as brown spot with concentric rings on leaf (Figure 1: A-B). Diseased samples were collected from four provinces of Thailand (Nonthaburi-2, Ratchaburi-1, Suphanburi-2 and Nakhonpathom-5). Leaf spot symptoms were cut into pieces of 5 mm, washed with sterilized 6% sodium hypochlorite (NaClO) for 1 min., soaked in sterilized water for 5 min and placed on Water Agar (WA) media plate (Deep *et al.*, 2014) and incubated at room temperature for 2-3 days. After incubation mycelial growth out of leaf piece. Hyphal tip was cut and placed on Potato Dextrose Agar (PDA) media plate until get pure culture.

Identification of pathogen

All isolates were identified by morphological characteristics of the colony appearance on PDA media plate and conidia. The conidia were determined by size (length and width) and characteristic of conidia (Deep *et al.*, 2014) using Nikon NIS-Element software and characteristic of conidia was observed under microscope.

Molecular identification was used to identify into species. The mycelia were placed on sterilized cellophane sheet, covered on PDA media for 12 days at room temperature. The colony was scraped off and crushed with liquid nitrogen by motar and pestle. The total powder of mycelia of 1-2 gram was put

into 2 ml tube. DNA was isolated according to Cetyl Trimethyl Ammonium Bromide (CTAB) method by Doyle and Doyle (1990) and Suksiri *et al.* (2018).

Polymerase chain reaction (PCR) was used primers ITS1 (3' TCC GTA GGT GAA CCT GCG G 5') and ITS4 (3' TCC TCC GCT TAT TGA TAT GC 5'). Amplification reactions were performed in a 25 µl mixture containing 0.5 mM MgCl₂, 0.2 mM of deoxynucleotide triphosphates, 0.8 µM of each primer, 300 ng/µl of DNA template, 1 U of *Taq* polymerase and 1X standard *taq* reaction buffer. PCR cycling conditions consisted of initial denaturation at 94 °C for 2 min. Thirty-five cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 2 min. Final extension at 72 °C for 10 min. Method modified according to White *et al.* (1990). PCR products were performed to sequence analysis by Bioneer company, Korea and identified species from database of National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tools (BLAST).

Antagonist

Pure culture of *Neosartorya hiratsukae* EU06, *N. pseudofischeri* EU13, *Neosartorya* sp. EU35, *Talaromyces muroii* EU07, *T. muroii* EU18 and *T. trachyspermus* EU23, *N. aureola* CHA01-A01, *N. fennelliae* CHA03-A11, *N. spinosa* CHA09-A01, and *T. muroii* CHA03-A03 were obtained from Soyong (2015), Suksiri and Poaim, Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Pathogenicity test

All isolates were tested pathogenicity by detached leaf bioassay. Kale was cultivated for 25 days and selected leaf with length of 15-20 cm, leaf was gently washed under running tap water, and wounded by sterilized needle (5 times). The agar plug (5 mm diameter) of *A. brassicicola* were placed on wound while control was used pure agar. The leaves were placed in moist chamber boxes. It was recorded diameter of lesion on 4 days. The experiments were conducted in completely randomized design (CRD). Each treatment consisted of three replicates.

Dual culture

The most virulent isolate from pathogenicity test was used for dual culture test, *Neosartorya* and *Talaromyces* against *A. brassicicola*. An agar plug (5 mm diameter.) of each antagonist and an agar plug of pathogen

obtained from the edge of 15 days old colony were placed 4 cm apart on opposite sides of a PDA plate (9 cm diameter). An agar plug of each antagonist and pathogen were placed on a separate PDA plate to control. The plates were incubated at room temperature for 20 days. The inhibition percent were calculated by using this formula: $[(x - y)/x] \times 100$, where x = amount of conidia of the plant pathogenic fungus in the control plate, and y = amount of conidia of plant pathogenic fungus in the dual culture plate. Experiment was conducted in completely randomized design (CRD). Each treatment consisted of four replicates.

Results

Isolation and identification of pathogen

Ten isolates were isolated from 30 leaf spot samples of kale. The color of colony and the conidia grown on PDA media showed little variation of colony color. The colony color of *A. brassicicola* isolates varied from light olive green to olivaceous black (Figure 1: C-D). The conidia of all isolates were same characteristics, conidia color was brown, obpyriform shape, chain and mostly had 4-6 transverse septa (Figure 1: E-J). The conidia size of *A. brassicicola* averaged length and width variation to each isolate include AbLK-NP01(Nakornpathom) 38.72 with 7.05 μm , AbLK-NP02 (Nakornpathom) 31.78 with 7.13 μm , AbLK-NP03 (Nakornpathom) 32.07 with 7.41 μm , AbLK-NP04 (Nakornpathom) 27.8 with 6.65 μm , AbLK-NP05 (Nakornpathom) 28.92 with 7.51 μm , AbLK-NB01 (Nonthaburi) 27.46 with 7.48 μm , AbLK-NB02 (Nonthaburi) 28.79 with 7.21 μm , AbLK-SP01 (Suphanburi) 30.77 with 7.25 μm , AbLK-SP02 (Suphanburi) 33.36 with 7.84 μm and AbLK-RB01 (Ratchaburi) 32.91 with 9.16 μm (Table 1).

Four isolates of AbLK-NP02 from Nakornpathom, AbLK-SP01 from Suphanburi, AbLK-RB01 from Ratchaburi and AbLK-NB01 from Nonthaburi were chosen to represent species identification. The result of molecular identification showed all isolates identity 99% of *A. brassicicola*, corresponding to accession number MF462311 and U05198 obtained to compare with data base in NCBI. That confirmed all isolates are *A. brassicicola*.

Pathogenicity test

Ten isolates of *A. brassicicola* were found to be virulence isolates by detach leaf bioassay. The result showed that isolated AbLK-NP01 was the

longest diameter of lesion at 20.75 mm, and followed by AbLK-NB01, AbLK-NP05, AbLK-NP02, AbLK-SP01, AbLK-SP02, AbLK-NP03, AbLK-RB01, AbLK-NB02 and AbLK-NP04 which diameter of lesion were 17.5, 16.56, 16.49, 16.12, 14.88, 14.27, 14.07, 11.57 and 10.19 mm., respectively (Figure 3). Characteristic of lesion was brown circle, and the scale of lesions changed the leaf color in virulence isolate which leaf changed to yellow, conversely the none virulence isolates showed a few change to yellow (Figure 2).

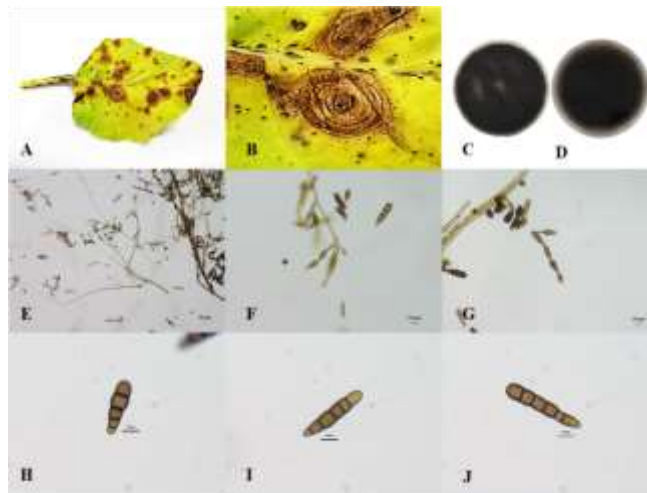


Figure 1. Leaf spot disease symptom and morphology of *A. brassicicola* (A) Leaf spot symptoms, (B) Characteristic of lesion, (C) Front of colony plate, (D) Back of colony plate, (E) Hypha and conidia of pathogen 10x magnification, (F) Conidiophore, (G) Hypha and conidia of pathogen 40x magnification, (H) Four transverse septa, (I) Five transverse septa and (J) Six transverse septa

Dual culture

Ten isolates of *Neosartorya* and *Talaromyces* showed actively against *A. brassicicola* AbLK-NP01. The result showed that *N. spinosa* CHA09-A01 (Figure 4) had the highest inhibited than other tested antagonists which the growth inhibition of 79.31% and number of conidia of 2.95×10^5 conidia/ml, and followed by *N. aureola* CHA01-A01, *Neosartorya* sp. EU35, *N. hiratsuka* EU06, *N. pseuifischeri* EU13, *N. fennelliae* CHA03-A11, *T. muroii* CHA03-A03, *T. muroii* EU18, *T. muroii* EU07 and *T. trachyspermus* EU23 which the growth inhibition of 78.60, 75.10, 72.99, 71.94, 60.02, 57.56, 50.55, 42.49 and 42.14%, respectively. The number of conidia were 3.05×10^5 , 3.55×10^5 , 3.85×10^5 , 4×10^5 , 5.7×10^5 , 6.05×10^5 , 7.05×10^5 , 8.2×10^5 and 8.25×10^5 conidia, respectively (Table 2).

Table 1. Morphology and province collection of *Alternaria brassicicola* isolated from leaf spot on kale

Isolates	Province of collection	Colony color on PDA	Conidial characteristics			
			Colour	Shape	Size of conidial (μm)	
					Length	Width
AbLK-NP01	Nakornpathom	Olive Gray	Brown	Obpyriform	38.72	7.05
AbLK-NP02	Nakornpathom	Dark Olive Green	Brown	Obpyriform	31.78	7.13
AbLK-NP03	Nakornpathom	Dark Olive Green	Brown	Obpyriform	32.07	7.41
AbLK-NP04	Nakornpathom	Dark Olive Green	Brown	Obpyriform	27.8	6.65
AbLK-NP05	Nakornpathom	Olive Gray	Brown	Obpyriform	28.92	7.51
AbLK-NB01	Nonthaburi	Light Olive Green	Brown	Obpyriform	27.46	7.48
AbLK-NB02	Nonthaburi	Light Olive Green	Brown	Obpyriform	28.79	7.21
AbLK-SP01	Suphanburi	Olivaceous Black	Brown	Obpyriform	30.77	7.25
AbLK-SP02	Suphanburi	Olivaceous Black	Brown	Obpyriform	33.36	7.84
AbLK-RB01	Ratchaburi	Light Olive Green	Brown	Obpyriform	32.91	9.16

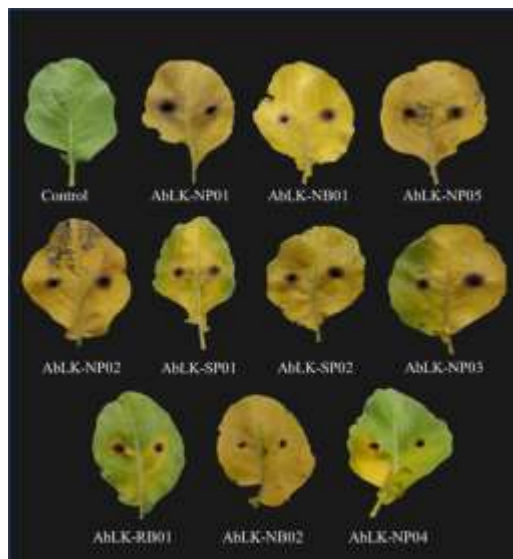


Figure 2. Characteristic lesion on kale leaf after 4 days inoculation with agar plug of *A. brassicicola* isolates

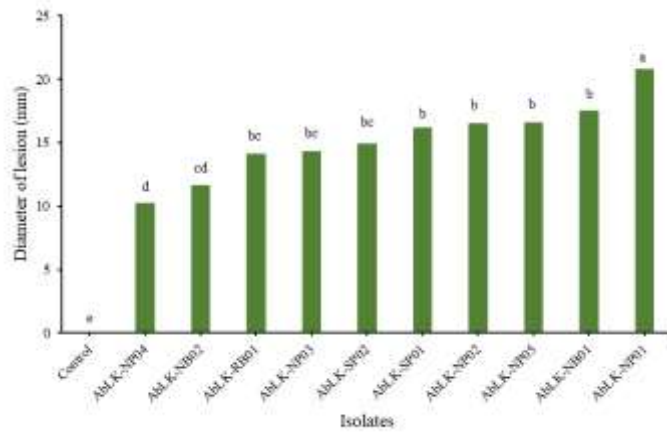


Figure 3. Pathogenicity test of *A. brassicicola* isolate by detach leaf bioassay. Different letter indicates significant differences ($p = 0.05$) between different treatments according to Duncan's Multiple Range Test

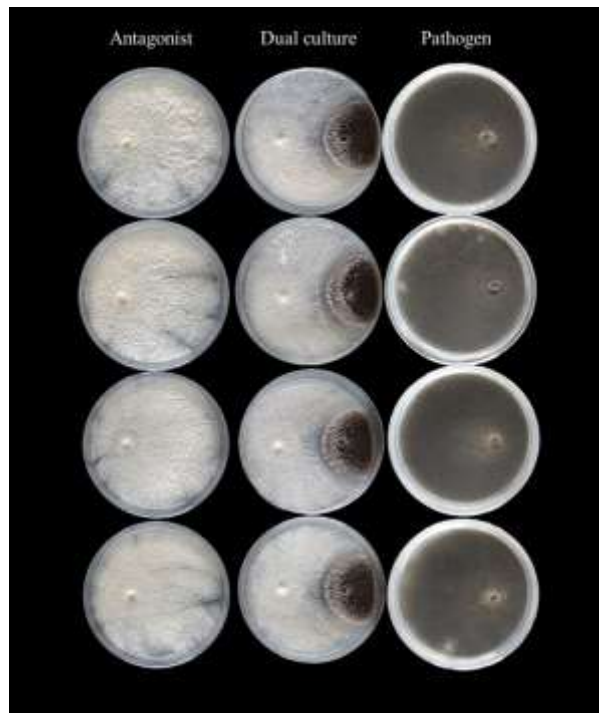


Figure 4. Characteristic colony dual culture of *N. spinosa* CHA09-A01 with *A. brassicicola* AblK-NP01.

Table 2. Dual culture of *Neosartorya* and *Talaromyces* for growth inhibition of *A. brassicicola* AbLK-NP01

Treatment	<i>Alternaria brassicicola</i>	
	Number of conidia/ml ($\times 10^5$)	Growth inhibition (%)
CHA09-A01	2.95	79.31 ^a
CHA01-A01	3.05	78.60 ^a
EU35	3.55	75.10 ^a
EU06	3.85	72.99 ^{ab}
EU13	4	71.94 ^{ab}
CHA03-A11	5.7	60.02 ^{bc}
CHA03-A03	6.05	57.56 ^c
EU18	7.05	50.55 ^{cd}
EU07	8.2	42.49 ^d
EU23	8.25	42.14 ^d
control	14.26	0 ^e

Different letter indicates significant differences ($p = 0.05$) between different treatments according to Duncan's Multiple Range Test

Discussion

The result of the present study showed ten isolates of *Alternaria brassicicola* isolated from leaf spot on kale in growing areas. The morphological of ten isolates showed no differed in colony color on PDA. The conidia color and conidia characteristic were not different in ten isolates. In part of conidia size was differed due to conidia of *A. brassicicola* are possible variation of length and width that was reported from Cho *et al.* (2001). It identified as *A. brassicicola* that similar to Deep *et al.* (2014). The molecular identification confirmed as *A. brassicicola*. The sequence from PCR product of four isolates were BLAST with database on NCBI. The result showed identity percentage not different and similarity sequence from report of Mahmoudi *et al.* (2017) and Claudia *et al.* (1995). This reason is confirmed other isolates are *A. brassicicola*. The pathogenicity test confirmed ten isolates were pathogenic isolates to cause leaf spot of kale. The pathogenic test was confirmed all isolates were pathogenic and caused leaf spot of kale (Figure 2). Each isolate was differed in virulence to disease (Figure 3) with a significant ($p = 0.05$). The

color on infested leaves with high virulent isolate turned yellow, conversely none virulence isolate made few yellow (Figure 2). The reason was possible *A. brassicicola* may produce toxin attack leaf according to report of Atsushi *et al.* (2009). The effective antagonist *Neosartorya* and *Talaromyces* against *A. brassicicola* was proved by dual culture technique. The result was observed a rank of effective antagonist *Neosartorya* (Table 2). In dual culture, the antagonist grew over colony of pathogen. The result showed *N. spinosa* CHA09-A01 (Figure 4) was highly effective as similar *Trichoderma* spp. against *A. brassicicola* reported Intana *et al.* (2005). Moreover, percentage inhibition of *N. spinosa* CHA09-A01 was high effective than those effective of volatile metabolites on *Trichoderma* spp. from literature searched (Amin *et al.*, 2010).

It concluded that the morphology was identified as *A. brassicicola* AbLK-NP01. *Neosartorya* and *Talaromyces* were proved to inhibit *A. brassicicola* by dual culture method. The result showed that *N. spinosa* CHA09-A01, *N. aureola* CHA01-A01, *Neosartorya* sp. EU35, *N. hiratsuka* EU06 and *N. pseudischi* EU13 had high effective to inhibit the tested pathogen of 79.31, 78.60, 75.1, 72.99 and 71.94%, respectively.

Acknowledgement

This work was supported by King Mongkut's Institute of Technology Ladkrabang (grant number A118-0261-010). The antagonists were supported by Supattra Poeaim, Mayamor Soyong and Supanan Suksiri. The samples were supported by farmers in Nonthaburi, Ratchaburi, Suphanburi and Nakornpathom province.

References

- Amin, F., Razdan, V. K., Mohiddin, F. A., Bhat, K. A. and Sheikh, P. A. (2010). Effect of volatile metabolites of *Trichoderma* species against seven fungal plant pathogens *in vitro*. *Journal of Phytology*. 2:34-37.
- Anan, K. and Athinuwat, D. (2016). Bio-formulation development from *Bacillus subtilis* TU-Orga1 for controlling the important diseases of chinese kale. *Journal of Science and Technology*. 5:794-812.
- Atsushi, M., Tajima, N., Higuchi, Y., Toyomasu, T., Sassa, T., Kato, N. and Dairi, T. (2009). Identification and functional analysis of brassicene C biosynthetic gene cluster in *Alternaria brassicicola*. *Bioorganic and Medicinal Chemistry Letters*. 19:870-874.
- Boonsang, N., Dethoup, T., Singburaudom, N., Gomes, N. and Kijjoa, A. (2014). *in vitro* antifungal activity screening of crude extracts of soil fungi against plant pathogenic fungi. *Biopesticides*. 7:156-166.
- Cho, H. S., Kim, B. R. and Yu, S. H. (2001). Taxonomic studies on *Alternaria* in Korea (1). *Mycobiology*. 29:27-42.

- Claudia, A., Victor, M., Lawrence, E. and Ginette, S. (1995). Comparison of nuclear ribosomal DNA sequences from *Alternaria* species pathogenic to crucifers. *Mycology Research*. 99:604-614.
- Deep, S., Sharma, P., Behera, N. and Chowdappa, P. (2014). Diversity in Indian isolates of *Alternaria brassicicola* (Schwein) Wiltshire causing black leaf spot disease in cauliflower. *Plant Pathology Journal*. 13:232-245.
- Dethoup, T., Kaewsalong, N., Songkumorn, P. and Jantasorn, A. (2018). Potential application of a marine-derived fungus, *Talaromyces tratensis* KUFA 0091 against rice diseases. *Biological Control*. 119:1-6.
- Doyle, J., Doyle, J. and Brown, A. (1990). A chloroplast-DNA phylogeny of the wild perennial relatives of soybean (*Glycine Subgenus glycine*): congruence with morphological and crossing groups. *Evolution*. 44:371-389.
- Iacomini, B., Avenot, H., Bataille, N., Laurent, E., Guenard, M. and Simoneau, P. (2004). In vitro fungicide sensitivity of *Alternaria* species pathogenic to crucifers and identification of *Alternaria brassicicola* field isolates highly resistant to both dicarboximides and phenylpyrroles. *Crop Protection*. 23:481-488.
- Intana, W., Suwanno, T. and Chamswang, C. (2005). Use of antifungal metabolite from *Trichoderma virens* for controlling chinese kale leaf spots caused by *Alternaria brassicicola*. *Walailak Journal of Science and Technology*. 2:1-9.
- Mahmoudi, R., Shams-Bakhsh, M. and Safaie, N. (2017). Phylogenetic analysis of *Alternaria raphani*, *A. arborescens* and *A. brassicicola* from canola in Iran. *Bioscience Biotechnology Research Communications*. 2:105-111.
- Pscheidt, J.W. and Ocamb, C. M. (2018). Cabbage and Cauliflower (*Brassica* sp.)-Damping-off (Wirestem). *Pacific Northwest Pest Management Handbooks*. pp. 1-2.
- Rimmer, R., Shattuck, V. and Buchwaldt, L. (2007). Compendium of *Brassica* diseases. *Hort Science*. 44:1509-1510.
- Siripanich, S. (2005). Situation and health problems from pesticide chemicals. Bureau of Epidemiology of Thailand. pp. 1-5.
- Suksiri, S., Laipasas, P., Soyong, K. and Poem, S. (2018). Isolation and identification of *Phytophthora* sp. and *Pythium* sp. from durian Orchard in Chumphon province, Thailand. *International Journal of Agricultural Technology*. 14:389-402.
- White, T. J., Bruns, T. D., Lee, S. B. and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A guide to methods and applications*. Academic Press, New York. pp. 315-322.

(Received: 27 August 2018, accepted: 24 October 2018)