Available online: www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not Bot Horti Agrobo, 2018, 46(2):630-638. DOI:10.15835/nbha46211076

Original Article

Effects of Culture Media on *Phytophthora palmivora* Growth, α-elicitin Production and Toxicity to *Dendrobium*

Apinya KHAIRUM, Oythip POOLSAWAT, Paniti PORNBUNGKERD, Akkawat THARAPREUKSAPONG, Sopone WONGKAEW, Piyada A. TANTASAWAT*

*Suranaree University of Technology, 111 University Avenue, Muang District, Nakhon Ratchasima 30000, Thailand; apinya_khan@hotmail.com; oythip@gmail.com; paniti_porn@sut.ac.th; akkawat@sut.ac.th; sopone@sut.ac.th; piyada@sut.ac.th (*corresponding author)*

Abstract

Four culture media were evaluated for their ability to induce *Phytophthora palmivora* growth and produce culture filtrate (CF), and to determine a CF concentration and culture period effective for *in vitro* screening of black rot resistance in *Dendrobium* cv. 'Earsakul'. Mycelial fresh weights of *P. palmivora* cultured in potato dextrose broth (PDB; the most commonly used medium for fungi), pea sucrose broth (PSB; a medium frequently used for *Phytophthora* spp.), and Murashige and Skoog broth (MSB; the most popular plant tissue culture medium) were found to be significantly higher than that in the newly developed modified oat meal broth (MOMB). When the total proteins of CFs were analysed with SDS-PAGE, a protein band of 10.5 kDa MW was found in CFs from all media with the highest level in PSB. LC-MS/MS analysis identified this protein as α-elicitin that had an identical amino acid sequence to the α-elicitin hibernalin of *P. hibernalis* and syringicin from *P. syringae*. The optimum conditions for *in vitro* selection of *Dendrobium* for black rot resistance using α-elicitin-containing CFs were also determined by evaluating the CF toxicity on *Dendrobium* protocorm-like bodies (PLBs) when cultured in all media supplemented with 0, 30, 50 and 100% CFs for seven, 14 and 21 d. The levels of PLB necrosis varied according to medium types, CF concentrations and culture periods. The maximum percentage of PLB necrosis (100%) was obtained in PSB supplemented with 50 and 100% CFs, and the severity of PLB necrosis was highest when treated with 100% CF for 14 and 21 d.

Keywords: black rot; *in vitro;* selection; mycelial growth; orchid; pea sucrose broth; toxin

Introduction

Black rot caused by *Phytophthora palmivora* is a serious disease of orchids worldwide. The disease is most frequently seen on *Cattleya* and their hybrids, but it also affects several orchid genera including *Dendrobium* (Orlikowski and Szkuta, 2006; Cating *et al*., 2010). Typical symptoms are observed mainly on leaves as black areas in different parts of plants. The optimal temperature for the oomycetal growth is 27.5-30 °C (Widmer, 2014), therefore it can spread widely in Thailand throughout the year. Several applications of systemic fungicides are often required to protect orchids against black rot; however, they are still limited by cost (McMillan *et al*., 2009). Therefore, orchid cultivars with black rot resistance are highly desirable.

In the last decade, many toxins produced during plant pathogenesis are known to be phytotoxic (Yoder, 1980). Phytotoxins are low molecular weight substances and secondary metabolites produced by plant pathogenic microorganisms (bacteria, oomycetes and fungi). They are toxic to plants and have been reported to act directly on protoplasts of the cells (Amusa, 2006). CFs of many phytopathogenic microorganisms are known to contain phytotoxic metabolites and toxins have been isolated from them. Moreover, the effects of phytotoxins against host tissues have also been evaluated *in vitro* in several species (Rao and Ramgoapl, 2010; Dehgahi *et al*., 2014; Jadon *et al*., 2015). Meanwhile, purified toxins as well as CFs from different pathogens including *Fusarium*, *Phytophthora* and *Alternaria* etc. were used for *in vitro* selection to obtain new resistant lines (Savita *et al*., 2011; Esmaiel *et al*., 2012; Lecomte *et al*., 2014). *Phytophthora* spp. have been reported

Received: 27 Oct 2017. Received in revised form: 19 Feb 2018. Accepted: 20 Feb 2018. Published online: 10 Mar 2018.

to secrete a variety of proteins including elicitins, *P. cactorum*-Fragaria protein (PcF), crinkling- and necrosis-inducing protein (CRN), cellulose-binding elicitor and lectin-like proteins, and necrosis- and ethylene-inducing-like protein (NLP) (Dong *et al*., 2012). Elicitins are 10 kDa extracellular proteins with 98 amino acids which can be divided into diverse classes including elicitins (class Iα [acidic elicitins], Iβ [basic elicitins] and II [highly acidic elicitin]) and elicitin-like proteins. They were found in *P. infestans*, *P. sojae*, *P. brassicae* and *P. ramorum* (Jiang *et al*., 2006). Plants recognize elicitin conserved structure known as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) by pattern recognition receptors (PRRs) (Zipfel and Robatzek, 2010; Derevnina *et al*., 2016; Uhlíková *et al*., 2016). Sensing of MAMPs or PAMPs lead to MAMP- or PAMP-triggered immunity (MTI or PTI) that can overcome microbe or pathogen infection by inducing primary defense responses and thus increase plant resistance to pathogens (Zipfel and Robatzek, 2010; Oßwald *et al*., 2014; Saubeau *et al*., 2014) Moreover, receptor-like protein ELR (elicitin response) is one of the first cell surface receptors known to mediate hypersensitive response (HR) (Du *et al*., 2015). Its biological activities involve induction of HR, systemic acquired resistance (SAR), phytoalexin, and pathogenesis related (PR) proteins as well as activation of necrotic response and electrolyte leakage (Capasso *et al*., 2001; Baillieul *et al*., 2003). It has been shown that α-elicitin of *P. plurivora* has dual roles in a susceptible interaction; association with the penetration of the pathogen by unknown mechanisms and suppression of important defense genes of host plants. The functions of α-elicitins may also be related to later stages of infection, especially sporulation and/or pathogen survival under saprophytic conditions. Therefore, elicitins appear to have conflicting functions, promoting virulence as well as serving as avirulent determinants by eliciting defense responses. Qualitative and quantitative differences in HR cell death induction and SAR indicated that the genetic basis of response to elicitin depend on variable plant taxa and types of elicitin. Thus, elicitins induce HR in some plant species including tobacco, potato, pepper, grapevine, citrus etc., whereas they exhibit lack of responsive capacity in other species e.g., tomato (Kamoun *et al*., 1993; Oßwald *et al*., 2014; Derevnina *et al*., 2016). However, the response of *Dendrobium* has not been studied.

In vitro selection using pathogenic toxins offers an effective means of screening for disease resistance in crop improvement programs because it allows greater environmental control, requires a smaller space, and is faster than conventional breeding (Rao and Ramgoapl, 2010; Savita *et al*., 2011). However, to effectively differentiate resistant and susceptible plants, a good selection system needs to be developed.

The objectives of this study were to evaluate the production of toxin-containing CF of *P. palmivora* on different oomycetal culture media and to determine an effective concentration of CF and the culture period to be used for screening black rot resistance in *Dendrobium* cv. 'Earsakul'. In addition, we identified the major protein secreted into CF as α-elicitin by using LC-MS/MS analysis.

Materials and Methods

Phytophthora palmivora culture and preparation of culture filtrates

Single-conidial cultures of *P. palmivora* isolate NK-53-9 (the most virulent isolate; Khairum *et al*., 2016) were maintained in potato dextrose agar (PDA; 20% (w/v) potato, 2% (w/v) dextrose and 2% (w/v) agar) under UV blacklight at 18-25 °C for 5 d. Two pieces (Φ = 1 cm) of a PDA with fungal mycelium were transferred to each flask containing 100 mL of four oomycetal culture media: potato dextrose broth [PDB; 20% (w/v) potato and 2% (w/v) dextrose; the most commonly used medium for fungi], pea sucrose broth [PSB; 12.5% (w/v) pea and 1% (w/v) sucrose; a medium frequently used for *Phytophthora* spp.], modified oat meal broth [MOMB; 5% (w/v) oat, 5% (w/v) rice bran, 5% (w/v) potato and 1% (w/v) sucrose; the newly developed medium for spore induction in our laboratory], and Murashige and Skoog broth [MSB; MS salts (Murashige and Skoog, 1962) and 2% (w/v) sucrose; the most popular plant tissue culture medium], and incubated with shaking at 100 rpm in the dark at 24-25 °C for 20 d. After incubation, the cultures were filtered through Whatman No.1 filter paper and the pH of the resulting CF was adjusted to 5.6 ± 0.2. The CF thus collected was filter-sterilized using 0.2 μm membrane filter discs and stored at -20 °C until required. The mycelium remaining on the filter paper was weighed to estimate oomycetal growth in each medium.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The CFs of *P. palmivora* were harvested from four oomycetal culture media (PDB, PSB, MOMB and MSB CFs) after 20 d. Controls were *P. palmivora*-free oomycetal culture media (PDB, PSB, MOMB and MSB). Protein electrophoresis was carried out under denaturing conditions according to the modified method of Laemmli (1970) by SDS-PAGE with 16% separating gel and 4% stacking gel. The samples were boiled for 5 min at 95 °C with a reducing sample buffer (containing 2-mercaptoethanol) and 3 μL of each sample were loaded in the gel. Electrophoresis was performed at 180 V for 90 min. Staining was performed by using 0.1% silver nitrate (Rockefeller University, 2016). The experiment was repeated four times with consistent results. A gel was stained by colloidal coomassie blue G250.

In-gel digestion and LC-MS/MS analysis

The protein band of interest was excised from the gel, and the band was cut into 2 mm^2 pieces and placed into a 1.5 mL microcentrifuge tube. For in-gel digestion, trypsin (Promega, WI, USA) solution was added and digested at 4 °C for 30 min. Gel pieces were incubated with 25 mM Ambic overnight at 37 °C. Peptides were extracted with 50% ACN and 1% formic acid, and dried with a centriVap centrifugal vacuum concentrator for 2 h, followed by reconstruction with buffer (2% ACN and 0.1% formic acid). Peptide identification was analyzed by liquid chromatography-mass spectrometer/ mass spectrometer (LC-MS/MS) analysis (Sharma *et al*., 2014; Rodpai *et al*., 2016).

Protein identification was performed by searching against the protein database from NCBIprot (Other Eukaryote) using MASCOT MS/MS Ion Search (http://www.matrixscience.com) with the initial searching parameters of Enzyme and Trypsin, which allowed up to three missed cleavages; carbamidomethylation as fixed modification, and oxidation (HW) and oxidation (M) as variable modifications; peptide mass tolerance of 0.5 Da and fragment mass tolerance of 0.6 Da; a peptide charge state of +1, +2, +3; instrument type (ESI-QUAD-TOF); and report top (Auto). The obtained peptide sequence from *P. palmivora* CFs was compared with the NCBI database which was restricted to the taxonomy of elicitin in *Phytophthora* species. Sequence identification of proteins was performed against known protein sequences in the NCBI database using the protein-protein basic local alignment search tool (BLASTp) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). These protein sequences were aligned by using the MEGA 7.0 program.

Assessment of culture filtrates for in vitro selection

PLBs (50 PLBs per treatment) of *Dendrobium* cv. 'Earsakul' were cultured together with CFs at final concentrations of 0, 30, 50 and 100% (v/v) in four oomycetal culture media (PDB, PSB, MOMB and MSB) and incubated with shaking at 50 rpm for seven, 14 and 21 d at 25 °C and a 12 h photoperiod.

Toxicity analysis

The toxic effects of the CFs were determined by the appearance of necrosis on PLBs. Two parameters were evaluated at seven, 14 and 21 d; the percentages of necrotic PLBs [(number of PLBs with a necrosis symptom/ total PLBs) × 100], and severity of necrosis (a 0-5 scale based on necrotic area per PLB (%); 0 = 0%, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80% and 5 = 81-100%).

Statistical analysis

A completely randomized design with five replications (one flask of ten PLBs per replication) was used. Data was analyzed using analysis of variance (ANOVA) of mycelial fresh weight, arcsine transformed percentage of necrotic PLBs and $(X+1)^{1/2}$ transformed severity of necrosis score. A mean comparison was performed by Duncan's multiple range test (DMRT) to evaluate the differences in ability to promote mycelial growth and toxin production of various CFs using the SPSS version 14.0 (Levesque and SPSS Inc., 2006).

Results and Discussion

Effects of oomycetal culture media on mycelium growth of Phytophthora palmivora

After culturing isolate NK-53-9 of *P. palmivora* which exhibited the highest virulence in *Dendrobium* detached leaf assay (Khairum *et al*., 2016) for 20 d in four different oomycetal culture media (PDB, PSB, MOMB and MSB), it was found that the fresh weights of mycelium cultured in four different oomycetal culture media were significantly different ($p < 0.05$). The highest mycelial fresh weight was observed in the PSB medium (4.89 g), but it was not significantly different from those cultured in PDB and MSB (4.29 g). However, mycelial growth was significantly lower in MOMB (1.6-fold; Table 1). The pea medium with added sucrose has also been reported to promote a high rate of mycelial growth of *P. cinnamomi* (Chee and Newhook, 1965a, 1965b). The *Phytophthora* species usually grow best on natural or synthetic media containing thiamine, a suitable carbon source such as sucrose, nitrogen sources, inorganic salts and minor elements (Erwin and Ribeiro, 1996). Sterols are not essential for vegetative growth, nevertheless, in a sterol-containing medium *Phytophthora* takes it up and enhances growth (dry weight or colony diameter) (Hendrix, 1964; Elliott, 1994). The main sterol constituents from pea seeds and oat grains were identified as sitosterol by chromatography (Ryan *et al*., 2007; Gangopadhyay *et al*., 2015). This sterol has proven its ability to enhance mycelial growth and could promote the most rapid growth of *P. infestants* (Dahlin *et al*., 2017).

Previous reports have also shown that oat meal agar (OMA) was optimal for mycelial growth of *P. palmivora* (Awuah and Frimpong, 2002), *P. infestans* (Sopee *et al*., 2012) and *P. megasperma* var. *sojae* (Ho, 1970). However, the MOMB medium appeared to promote the least growth of *P. palmivora* in our system (Table 1). Because of the high cost of oats, this medium was modified by supplementing with potato and rice bran. Natural media containing potato tuber, which contains suitable sources of nitrogen, mineral salts and growth factors, and sugar was found to be appropriate for the growth of most fungi (Beever and Bollard, 1970; Sumbali, 2010). Meanwhile rice bran, a by-product of rice milling, is the best source of phytosterols, especially ß-sitosterol (the major sterol in rice bran) (Derakhshan-Honaroarvar *et al*., 2010; Özdestan *et al*., 2014). However, several researchers reported that rice bran contains bioactive compounds (e.g., phenolics, proteins) that could inhibit fungal growth, biomass and mycotoxigenic potential in some pathogenic fungi such as *Rhyzopus oryzae* (Souza *et al*., 2010), *Colletotrichum gloeosporioides* (Phungamngoen and Sungsri-in, 2011) and *Fusarium graminearum* (Fernanda *et al*., 2013). The presence of these bioactive compounds may negatively affect growth of *P. palmivora* in MOMB.

Identification of secreted protein in culture filtrates of Phytophthora palmivora and effects of culture media on its production

In the present investigation, *P. palmivora* was found to secrete a protein into the oomycetal culture media. When the total proteins from the obtained CFs were separated based on molecular weight using SDS-PAGE, the silver staining clearly revealed a major protein band with ca. 10.5 kDa MW. This protein was not found in *P. palmivora*-free control, but was present in all *P. palmivora* CFs at different quantities PSB > PDB > MOMB > MSB (Fig. 1). The MW of this protein is comparable to elicitins that were previously reported in sexual *Phytopthora* spp. (Churngchow and Rattarasarn, 2000). In CF of *P. palmivora*, the causal agent of rubber, palmivorein, a small secreted protein of ca. 10 kDa MW in the elicitin family,

Table 1. Similarity search between α-elicitin hibernalin protein sequence and accessions in NCBI database using BLASTp

Proteins	E-value[a]	Identity/ Query cover (%)
α-elicitin syringicin [*P. syringae*; P85436.1][b]	7e-62	100/100
α-elicitin INF1 [*P. capsici*; AFY98083.1]	7e-60	97/100
Sojein 1 protein [*P. sojae*; CAA07710.1]	3e-59	96/100
Sojein 4 protein [*P. sojae*; CAA07713.1]	4e-58	95/100
α-elicitin, infestin [*P. infestans*, peptide 98 aa; AAB31120.1]	5e-58	93/100
Sojein 3 protein [*P. sojae*; CAA07712.1]	2e-57	93/100
α-elicitin MGM-α [*P. megasperma*; P35689.1]	3e-57	93/100
Sojein 2 protein [*P. sojae*; CAA07711.1]	3e-56	92/100
α-elicitin DRE-α [*P. drechsleri*; P35696.1]	1e-54	89/100
ß-elicitin DRE- ß [*P. drechsleri*; P35697.1]	1e-52	86/100
Chain A, structure of fungal elicitor, nmr, 18 structures [*P. cryptogea*; 1BEG_A]	3e-51	84/100
ß-elicitin cryptogein; Short=CRY [*P. cryptogea*; P15570.2]	4e-51	84/100
Chain A, ß-cryptogein-chloresterol complex [*P. cryptogea*; 1LRI_A]	8e-51	83/100
Elicitin [*P. sojae*; XP_009532175.1]	2e-50	82/100
ß-megaspermin [*P. megasperma*; CAD38502.1]	2e-50	83/100
ß-elicitin cinnamomin [*P. cinnamomi*; P15569.1]	3e-50	83/100
Highly acidic elicitin [*Phytophthora* × *multiformis*; AAY85663.1]	6e-50	82/100
Cinnamomin [*P. cinnamomi*; CAB38321.1]	6e-50	83/100
Highly acidic elicitin [*P. cinnamomi*; CAB38324.1]	1e-49	83/100
Hypothetical protein PHYSODRAFT_355153 [*P. sojae*; XP_009532176.1]	8e-49	81/100
Basic elicitin partial [*P. uniformis*; ABM53007.1]	2e-48	80/100
Basic elicitin partial [*Phytophthora* × *alni*; ABM52996.1]	2e-48	80/100
Basic elicitin partial [*Phytophthora* × *multiformis*; ABM53003.1]	2e-48	80/100
α-elicitin 2 [*P. lateralis*; ABG75610.1]	2e-48	80/100
Basic elicitin [*Phytophthora* × *multiformis*; AAY85665.1]	3e-48	80/100
Basic elicitin [*Phytophthora* × *alni*; AAY85677.1]	1e-47	79/100
Acidic elicitin [*P. palmivora*; AJE68929.1]	6e-51	93/88

[a]Expected value (*E*-value) refer to the number of matches expected by chance alone. The lower the *E*-value, the more strongly supported the match.
[b]NCBI accession number.

was isolated and has been shown to cause necrosis on tobacco and rubber leaves (Churngchow and Rattarasarn, 2000). It is interesting to note that appreciable differences in an extracellular 10.5 kDa protein quantities were observed among CFs that were harvested from four oomycetal culture media, suggesting the differential effects of various media on *P. palmivora* protein production and/or secretion.

When the secreted protein was identified by LC-MS/MS, it showed the presence of LMCASTACK peptide, which was found to significantly match α-elicitin hibernalin and syringicin proteins from *P. hibernalis*, the causal pathogen of citrus lemon brown rot and *P. syringae*, the causal pathogen of citrus fruit rot, respectively. Its MW was estimated by LC-MS/MS as ca. 10.536 kDa with 4.68 pI, which is very close to the three α-elicitins, named hibernalin1, hibernalin 2 and hibernalin 3 (hib1, hib2 and hib3 with MW of 10.19, 10.21 and 10.22 kDa, respectively) found in *P. hibernalis* Carne 1925 CF, and close to the 10.19 kDa MW of syringicin (Capasso *et al*., 2001; Capasso *et al*., 2008). Hib1 was shown to be active in both the hypersensitivity response and electrolyte leakage assays. Furthermore, it was found to induce defense responses in resistant potato plants against *P. infestans*, for example, increasing expression of glucose oxidase, NADPH oxidase, superoxide dismutase, glutathione reductase, catalase and peroxidase enzymes (Saubeau *et al*., 2014). These results suggest that these elicitins may play an important role in host-pathogen interactions.

When one hundred protein sequences from 22 *Phytophthora* spp. from the NCBI database were compared, it was shown that 28 of these protein sequences shared high homology to α-elicitin hibernalin and syringicin (supported by *E*-value close to 0) including acidic elicitin from *P. palmivora* infecting rubber (Table 1). Although these elicitins were isolated from different *Phytophthora* species infecting different host plants (Capasso *et al*., 2008). These results suggest that particular elicitin member(s) may be conservative in diverse *Phytophthora* species, possibly possessing crucial functions in pathogenesis. Phylogenetic analysis based on the mature protein sequences of the 28 elicitins from 22 *Phythophthora* spp. separated them into three major clusters, cluster I, II and III. Fourteen were grouped into cluster I. This cluster was divided into two subclusters (IA and IB). Subcluster IA consisted of five β-elicitins and one hypothetical protein from five *Phythophthora* spp. infecting several plants (*Phytophthora* × *multiformis*, *Phytophthora* × *alni*, *P. uniformis*, *P. sojae* and *P. cinnamomi*). Subcluster IB consisted of two α-elicitins

and β-elicitins from three other *Phythophthora* spp. (*P. drechsleri*, *P. megasperma* and *P. cryptogea*). Cluster II consisted of nine α-elicitin (α-elicitin hibernalin, α-elicitin syringicin, α-elicitin INF1, α-elicitin MGM-α, acidic elicitin sojein 1 protein, sojein 4 protein, sojein 3 protein and sojein 2 protein) from *P. hibernalis, P. syringae, P. capsici*, *P. palmivora*, *P. megasperma* and *P. sojae* in subcluster IIA and one separated individual from *P. infestans* (α-elicitin, infestin). By contrast, cluster III consisted of four α-elicitins from *P. sojae*, *Phytophthora* × *multiformis*, *P. cinnamomi* and *P. lateralis* (Fig. 2). These results suggest that there is no relationship between these elicitin amino acid sequences based on either pathogen species or host plants.

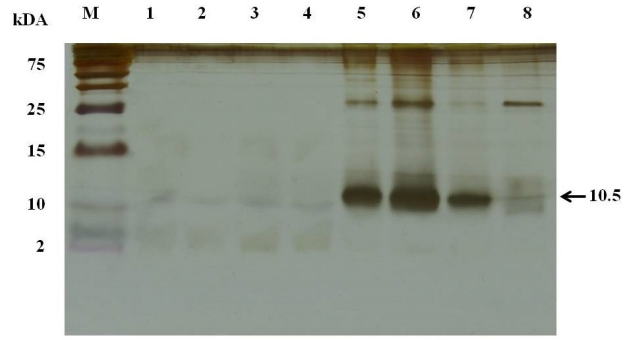

Fig. 1. SDS-PAGE of extracellular proteins secreted by *P. palmivora* after being cultured for 21 d. Standard proteins (M), four controls (*P. palmivora*-free oomycetal culture media; PDB, PSB, MOMB and MSB; lanes one to four, respectively) and *P. palmivora* CFs on four different oomycetal culture media (PDB, PSB, MOMB and MSB; lanes five to eight, respectively).

Effects of oomycetal culture media on toxicity of culture filtrate and determination of optimum conditions for in vitro selection of Dendrobium protocorm-like bodies (PLBs)

In order to obtain a suitable oomycetal culture medium for the production of *P. palmivora* CF with maximum efficiency for *in vitro* selection of black rot resistance in *Dendrobium*, four different oomycetal culture media (PDB, PSB, MOMB and MSB), four different concentrations of CF [0 (control), 30, 50 and 100%] and three culture periods (seven, 14 and 21 d) were evaluated. *In vitro* selection of *Dendrobium* PLBs in α-elicitin-containing CFs showed that the effects of medium types were highly significant ($p < 0.01$) on percentages of necrotic PLBs and severity of

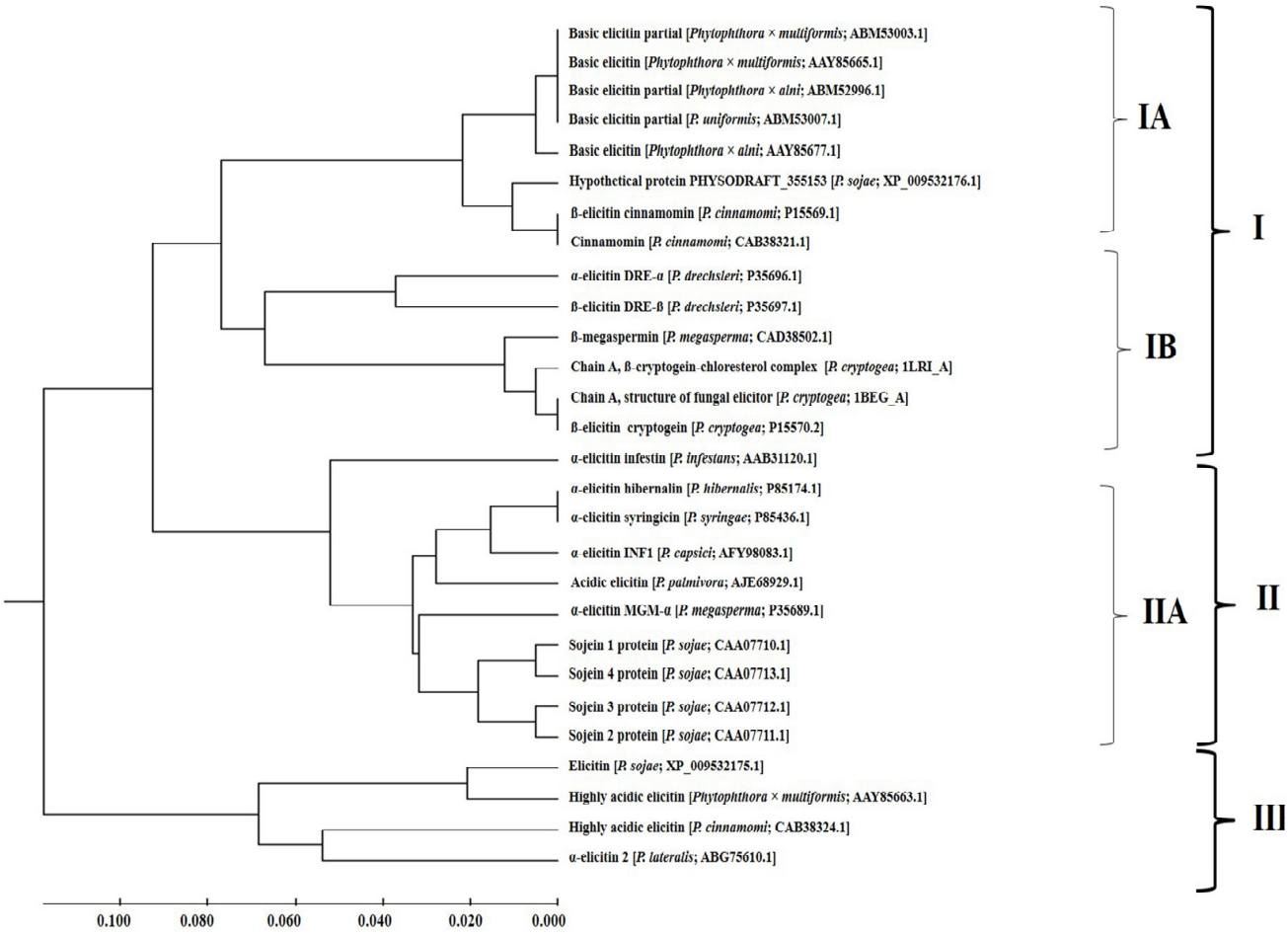

Fig. 2. Phylogenetic analysis of α-elicitins from different *Phytophthora* spp. with α-elicitin hibernalin (*P. hibernalis*) based on the mature protein sequences.

necrosis. The highest toxicity was obtained in the PSB medium with an average percentage of necrotic PLBs of 65.09% and an average severity of necrosis score of 1.31, which were significantly higher than those of other media, particularly MSB (Table 2). Different concentrations of CFs also affected percentages of necrotic PLBs and severity of necrosis significantly ($p < 0.01$). PLB necrosis increased as CF concentrations increased. The most effective CF concentration for *in vitro* selection was 100% with an average percentage of necrotic PLBs of 76.14% and an average severity of necrosis score of 1.43. Nevertheless, the reduction of CF concentration from 100 to 50% decreased the percentage of necrotic PLBs by only 13% (Table 3). Among the three culture periods used in this study, there were no significant differences ($p > 0.05$) on percentages of necrotic PLBs. However, a highly significant difference ($p < 0.01$) was observed among the culture periods on severity of necrosis. It was shown that necrosis increased as the culture period increased, and the highest average severity of necrosis score (0.99) was reached at 21 d (Table 4). When all three

Table 2. Effects of four different oomycetal culture media on fresh weight of *Phytophthora palmivora* mycelium at 20 d after culture

Oomycetal culture media	Fresh weight of mycelium (g)
PDB	4.29 ± 0.38 a[a]
PSB	4.89 ± 0.20 a
MOMB	3.11 ± 0.55 b
MSB	4.29 ± 0.10 a

[a] Data are presented as means ± SE. Means in the same column with different letters are significantly different (Duncan's multiple range test (DMRT), $p < 0.05$).

Table 3. Effects of four different oomycetal culture media of *Phytophthora palmivora* on percentages of necrotic protocorm-like bodies (PLBs) and necrosis severity of *Dendrobium* cv. 'Earsakul'

Oomycetal culture media	Necrotic PLBs (%)	Severity of necrosis[b]
PDB	56.11 ± 6.00 b[a]	0.77 ± 0.04 b
PSB	65.09 ± 6.11 a	1.31 ± 0.06 a
MOMB	55.96 ± 5.48 b	0.74 ± 0.04 b
MSB	8.43 ± 3.02 c	0.09 ± 0.01 c

[a] Data are presented as means ± SE. Means in the same column with different letters are significantly different (Duncan's multiple range test (DMRT), $p < 0.05$).
[b] Severity of necrosis score (0-5 scale based on necrotic area per PLB (%); 0 = 0%, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80% and 5 = 81-100%).

Table 4. Effects of four different concentrations of culture filtrates of *Phytophthora palmivora* on percentages of necrotic protocorm-like bodies (PLBs) and necrosis severity of *Dendrobium* cv. 'Earsakul'

Concentrations of culture filtrate (%)	Necrotic PLBs (%)	Severity of necrosis[b]
0 (Control)	0.00 ± 0.00 d[a]	0.00 ± 0.00 d
30	46.20 ± 5.72 c	0.61 ± 0.04 c
50	66.25 ± 5.63 b	0.93 ± 0.03 b
100	76.14 ± 4.86 a	1.43 ± 0.05 a

[a] Data are presented as means ± SE. Means in the same column with different letters are significantly different (Duncan's multiple range test (DMRT), $p < 0.05$).
[b] Severity of necrosis score (0-5 scale based on necrotic area per PLB (%); 0 = 0%, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80% and 5 = 81-100%).

Table 5. Effects of three culture periods on percentages of necrotic protocorm-like bodies (PLBs) and necrosis severity of *Dendrobium* cv. 'Earsakul'

Culture periods (d)	Necrotic PLBs (%)	Severity of necrosis[b]
7	42.89 ± 5.14[a]	0.50 ± 0.02 c
14	48.06 ± 5.34	0.77 ± 0.04 b
21	51.27 ± 5.53	0.99 ± 0.05 a

[a] Data are presented as means ± SE. Means in the same column with different letters are significantly different (Duncan's multiple range test (DMRT), $p < 0.05$).
[b] Severity of necrosis score (0-5 scale based on necrotic area per PLB (%); 0 = 0%, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80% and 5 = 81-100%).

Table 6. Percentages of necrotic protocorm-like bodies (PLBs) and necrosis severity of *Dendrobium* cv. 'Earsakul' cultured in PSB medium supplemented with various culture filtrate concentrations at different culture periods

Culture filtrate concentrations (%)	Culture periods (d)	Necrotic PLBs (%)	Severity of necrosis[b]
0 (control)	7	0.00 ± 0.00 c[a]	0.00 ± 0.00 g
	14	0.00 ± 0.00 c	0.00 ± 0.00 g
	21	0.00 ± 0.00 c	0.00 ± 0.00 g
30	7	64.00 ± 20.15 b	0.64 ± 0.07 f
	14	68.00 ± 19.60 ab	1.08 ± 0.16 e
	21	70.00 ± 18.44 ab	1.26 ± 0.17 e
50	7	100.00 ± 0.00 a	1.08 ± 0.04 e
	14	100.00 ± 0.00 a	1.45 ± 0.09 d
	21	100.00 ± 0.00 a	2.08 ± 0.08 b
100	7	100.00 ± 0.00 a	1.78 ± 0.13 c
	14	100.00 ± 0.00 a	3.10 ± 0.10 a
	21	100.00 ± 0.00 a	3.40 ± 0.11 a

[a] Data are presented as means ± SE. Means in the same column with different letters are significantly different (Duncan's multiple range test (DMRT), $p < 0.05$).
[b] Severity of necrosis score (0-5 scale based on necrotic area per PLB (%); 0 = 0%, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80% and 5 = 81-100%).

factors were considered, the maximum percentage of necrotic PLB (100%) was obtained in the PSB medium supplemented with 50 and 100% CFs for seven, 14 and 21 d. Severity of necrosis on PLBs was the highest when treated with 100% CF in PSB medium for 14 and 21 d, however, the necrotic symptoms became evident at a lower concentration (50 % CF) after 21 d (Table 5). PLBs selected in PSB medium at 21 d also appeared more necrotic (brown and black) than PLBs selected in other oomycetal culture media at the same concentrations of CFs. By contrast, control PLBs (0% CF) remained green in all culture media throughout the experiment (data not shown).

Our results reveal that the PSB CF at a concentration of 50% selected for 21 d is the most suitable condition for efficient *in vitro* selection for black rot resistance in *Dendrobium* since it requires the lowest CF concentration that allows 100% of necrotic PLBs (Table 6). Under this condition, most of the necrotic PLBs died after being transferred to a CF-free medium, allowing selection of the mutant PLBs that are toxicity resistant which can survive and develop into plantlets.

Using this condition for *in vitro* selection of *Dendrobium* cv. 'Earsakul' PLBs, we were able to obtain several putative mutants that were resistant to *P. palmivora* in the detached leaf assay (Khairum *et al*., 2016; Khairum *et al*., unpublished data). Similarly, the PSB medium was successfully used to obtain CF of *P. parasitica* for *in vitro* selection of *Citrus jambhiri* for resistance to root and crown rot as well as foot rot and brown rot of fruits (Savita *et al*., 2011). However, in *P. capsici*, CFB was optimal for toxin production (Qi *et al*., 2006). Our results on the optimal culture period (21 d) are in good agreement with a report from Behnke (1979) which showed that most of the potato calli became brownish and stopped growing in toxic media after three weeks. The phytotoxicity effects of toxins and CFs as well as their usefulness for *in vitro* selection have also been demonstrated in other plant-pathogen systems (Savita *et al*., 2011; Dehgahi *et al*., 2014; Valencia *et al*., 2014). However, as far as we know, we are the first to report the usage of *P. palmivora* CF for *in vitro* selection in *Dendrobium*.

It is interesting to note that the PSB medium is also suitable for mycelial growth of *P. palmivora* resulting in the highest fresh weight of mycelium (Table 2). MSB and PDB were able to induce comparable mycelial growth with PSB, but they produced lower toxin levels than PSB, as suggested by significantly lower percentages of necrotic PLBs and severity of necrosis (Tables 2 and 3). Necrosis and plant cell death were correlated with genetic basis of response to toxic metabolites or secreted proteins including elicitin from *Phytophthora* spp., which was variable depending on plant taxa. It is possible that the α-elicitin caused tissue necrosis of *Dendrobium* PLBs since its levels in CFs appear to correlate well with the extent of necrotic responses, however, this needs to be elucidated by future experiment with purified α-elicitin. Oomycete growth and sporulation *in vitro* is influenced by sterol contents and is highly correlated with sterol binding, especially in *Phytophthora* and *Pythium* spp. (Osman *et al*., 2001; Stong *et al*., 2013). Elicitin can act as sterol carriers by scavenging sterols from plasma membranes of host cells. Upon sterol binding, elicitin was initially suggested to disrupt plasma membrane integrity, leading to induced cell death (Derevnina *et al*., 2016). And it has also been reported that only a sterol-elicitin complex binds to a plasmalemma receptor and triggers the biological responses of plants (Ponchet *et al*., 1999; Osman *et al*., 2001). Although recent findings suggested that sterol binding may be independent from defense elicitation (Dokladal *et al*., 2012; Derevnina *et al*., 2016; Uhliková *et al*., 2016), its involvement may vary with plant species. Natural additives (legumes, vegetables and cereals) in oomycetal culture media are the main sources of plant sterols/ phytosterols, which are known to be structural components of plant cell membrane (Moreau *et al*., 2002; Lagarda *et al*., 2006). High total plant sterols (1,337 mg kg^{-1} dry weight) were measured in pea and lower contents (246 mg kg^{-1} dry weight) were found in potato (Piironen *et al*., 2003). In whole grain oats, the total sterol contents were 350-491 mg kg^{-1} dry weight of kernel (Piironen *et al*., 2002). A phytosterol-enriched constituent in PSB may enhance the production of activated sterol-loaded elicitin, which induces severe necrotic development in *Dendrobium* cv. 'Earsakul' PLBs. These hypotheses are supported by our results, which revealed that MSB (synthetic media without natural ingredients) promoted mycelial growth, but induced the lowest severity of necrosis (Tables 2 and 3). Interestingly, this medium also induced the lowest quantity of α-elicitin (Fig. 1).

Conclusions

We are the first to report a new α-elicitin in CF of *P. palmivora*, the causal agent of *Dendrobium* black rot, which significantly matches α-elicitin hibernalin from *P. hibernalis* and syringicin from *P. syringae*, sharing a high homology to several elicitins from diverse *Phytophthora* spp. infecting different host plants. Our results suggest that PSB is the most suitable medium for the promotion of *P. palmivora* growth, for the production of α-elicitin and for inducing necrosis in *Dendrobium* cv. 'Earsakul' PLBs, therefore it can be used to produce CF efficient for screening of black rot resistance. The concentrations of *P. palmivora* CF and culture periods also affected PLB necrosis, and the most optimized condition for effective selection of resistant *Dendrobium* PLBs was 50% of CF selected after 21 d. These results are crucial for the future development of black rot resistant *Dendrobium*.

Acknowledgements

The authors would like to thank Dr. Saengduen Moonsom, Dr. Watcharin Yuttavanichakul, Kanravee Issarangkul Na Ayuthaya, Supaporn baiya and Sukunya Chaipayang for their technical help and guidance with LC-MS/MS analysis. We are very grateful to Peter Charles Bint for editing this manuscript. This work was supported by the Office of the Higher Education Commission under the NRU project of Thailand and grants from the Thailand Research Fund and Suranaree University of Technology, Thailand.

References

Amusa NA (2006). Microbially produced phytotoxins and plant disease management. African Journal of Biotechnology 5:405-414.

Awuah RT, Frimpong M (2002). Cocoa-based media for culturing *Phytophthora palmivora* (Butl.) Butl., causal agent of black pod disease of cocoa. Mycopathologia 155:143-147.

Baillieul F, de Ruffray P, Kauffmann S (2003). Molecular cloning and biological activity of α, β, and γ-megaspermin, three elicitins secreted by *Phytophthora megasperma* H20. Plant Physiology 131:155-166.

Beever RE, Bollard EG (1970). The nature of the stimulation of fungal growth by potato extract. Journal of General Microbiology 60:273-279.

Behnke M (1979). Selection of potato callus for resistance to culture filtrates of *Phytophthora infestans* and regeneration of resistant plants. Theoretical and Applied Genetic 55:69-71.

Capasso R, Cristinziob G, Di Maroc A, Ferrantid P, Parentee A (2001). Syringicin, a new alpha-elicitin from an isolate of *Phytophthora syringae*, pathogenic to citrus fruit. Phytochemistry 58:257-262.

Capasso R, Di Maro A, Cristinzio G, De Martino A, Chambery A, Daniele A, … Parente A (2008). Isolation, characterization and structure–elicitor activity relationships of hibernalin and its two oxidized forms from *Phytophthora hibernalis* Carne 1925. Journal of Biochemistry 143:131-141.

Cating RA, Palmateer AJ, Stiles CM, Rayside PA (2010). Black rot of orchids caused by *Phytopthora cactorum* and *Phytophthora palmivora* in Florida. Plant Health Progress doi: 10.1094/PHP-2010-0614-01-DG.

Chee C-H, Newhook FJ (1965a). Improved methods for use in studies on *Phytophthora cinnamomi* Rands and other *Phytophthora* species. New Zealand Journal of Agricultural Research 8:88-95.

Chee C-H, Newhook FJ (1965b). Nutritional studies with *Phytophthora cinnamomi* Rands. New Zealand Journal of Agricultural Research 8:523-529.

Churngchow N, Rattarasarn M (2000). The elicitin secreted by *Phytophthora palmivora*, a rubber tree pathogen. Phytochemistry 54:33-38.

Dahlin P, Srivastava V, Ekengren S, McKee LS, Bulone V (2017). Comparative analysis of sterol acquisition in the oomycetes *Saprolegnia parasitica* and *Phytophthora infestans*. PLoS One 12(2), e0170873.

Dehgahi R, Zakaria L, Joniyas A, Subramaniam S (2014). *Fusarium proliferatum* culture filtrate sensitivity of *Dendrobium* sonia-28's PLBs derived regenerated plantlets. Malaysian Journal of Microbiology 10:241-248.

Derakhshan-Honaroarvar M, Hamedi MM, Pirouzifard MKh (2010). Rice bran phytosterols of three widespread Iranian cultivars. Journal of Agricultural Science and Technology 12:167-172.

Derevnina L, Dagdas FY, De la Concepcion JC, Bialas A, Kellner R, Petre B, … Kamoun S (2016). Nine things to know about elicitins. New Phytologist 212:888-895.

Dokladal L, Oboril M, Stejskal K, Zdrahal Z, Ptackova N, Chaloupkova R, … Lochman J (2012). Physiological and proteomic approaches to evaluate the role of sterol binding in elicitin-induced resistance. Journal of Experimental Botany 63:2203-2215.

Dong S, Kong G, Qutob D, Yu X, Tang J, Kang J, … Wang Y (2012). The NLP toxin family in *Phytophthora sojae* includes rapidly evolving groups that lack necrosis-inducing activity. Molecular Plant-Microbe Interactions 25:896-909.

Du J, Verzaux E, Chaparro-Garcia A, Bijsterbosch G, Keizer LCP, Zhou J, … Vleeshouwers VG (2015). Elicitin recognition confers enhanced resistance to *Phytophthora infestans* in potato. Nature Plants 1:15034.

Elliott CG (1994). Reproduction in fungi: genetical and physiological aspects. Chapman and Hall, London.

Erwin DC, Ribeiro OK (1996). Phytophthora disease worldwide. American Phytopathological Society, Minnesota.

Esmaiel NM, Al-Doss AA, Barakat MN (2012). *In vitro* selection for resistance to *Fusarium oxysporum* f. sp. *dianthi* and detection of genetic polymorphism via RAPD analysis in carnation. Journal of Medicinal Plants Research 6:3997-4004.

Fernanda AP, Cristiana CB, Sílvia LRM, Jaqueline GB, Eliana BF (2013). Activity of rice bran protein extracts against *Fusarium graminearum*. African Journal of Agricultural Research 8:6283-6290.

Gangopadhyay N, Hossain MB, Rai DK, Brunton NP (2015). A review of extraction and analysis of bioactives in oat and barley and scope for use of novel food processing technologies. Molecules 20:10884-10909.

Hendrix JW (1964). Sterol induction of reproduction and stimulation of growth of *Pythium* and *Phytophthora*. Science 144:1028-1029.

Ho HH (1970). A study of growth and sporulation of *Phytophthora megasperma* var. *sojae*. Sydowia 24:51-58.

Jadon KS, Shah R, Gour HN, Sharma P (2015). Effect of environmental factors on toxin production of *Drechslera bicolor*, a causal agent of leaf blight in bell pepper. African Journal of Microbiology Research 9:521-526.

Jiang RHY, Tyler BM, Whisson SC, Hardham AR, Govers F (2006). Ancient origin of elicitin gene clusters in *Phytophthora* genomes. Molecular Biology and Evolution 23:338-351.

Kamoun S, Young M, Glascock C, Tyler BM (1993). Extracellular protein elicitors from *Phytophthora*: host-specificity and induction of resistance to fungal and bacterial phytopathogens. Molecular Plant-Microbe Interactions 6:15-25.

Khairum A, Tharapreuksapong A, Wongkaew S, Tantasawat PA (2016). Cultural characteristics and pathogenicity analysis of *Phytophthora palmivora*, causal pathogen of black rot in orchids. In: Rahman MA, Dasic P (Eds). Proceedings of the International Conference on Agricultural, Food, Biological and Health Sciences (AFBHS-16). Kuala Lumpur, Malaysia pp 101-106.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lagarda ML, García-Llatas G, Farré R (2006). Analysis of phytosterols in foods. Journal of Pharmaceutical and Biomedical Analysis 41:1486-1496.

Lecomte M, Hamama L, Voisine L, Gatto J, Hélesbeux J-J, Séraphin D, … Berruyer R (2014). Partial resistance of carrot to *Alternaria dauci* correlates with *in vitro* cultured carrot cell resistance to fungal exudates. PLoS One 9(7), e101008.

Levesque R, SPSS Inc. (2006). SPSS programming and data management. SPSS Institute (3rd ed), New York.

McMillan RT, Jr, Palmateer A, Cating RA (2009). Problems in controlling *Phytopthora cactorum* on *Cattleya* orchids. Proceedings of the Florida State Horticultural Society 122:426-428.

Moreau RA, Whitaker BD, Hicks KB (2002). Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Progress in Lipid Research 41:457-500.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-496.

Orlikowski LB, Szkuta G (2006). *Phytophthora* rot of some orchids - new disease in Poland. Phytopathologia Polonica 40:57-61.

Osman H, Vauthrin S, Mikes V, Milat M-L, Panabières F, Marais A, … Blein J-P (2001). Mediation of elicitin activity on tobacco is assumed by elicitin-sterol complexes. Molecular Biology of the Cell 12:2825-2834.

Özdestan Ö, Erol T, Acar A (2014). Phytosterols in rice bran and usage of rice bran in food industry. In: Straumite E (Ed). Proceedings of 9th Baltic Conference on Food Science and Technology "Food for Consumer Well-Being" FoodBalt 2014, Jelgava pp 24-27.

Oßwald W, Fleischmann F, Rigling D, Coelho AC, Cravador A, Diez J, … Werres S (2014). Strategies of attack and defence in woody plant – *Phytophthora* interactions. Forest Pathology 44:169-190.

Phungamngoen A, Sungsri-in M (2011). Antifungal activity of rice bran extracts against *Colletotrichum gloeosporioides* isolated from fresh and fresh-cut mango. Agricultural Science Journal 42:323-325.

Piironen V, Toivo J, Lampi A-M (2002). Plant sterols in cereals and cereal products. Cereal Chemistry 79:148-154.

Piironen V, Toivo J, Puupponen-Pimiä R, Lampi A-M (2003). Plant sterols in vegetables, fruits and berries. Journal of the Science of Food and Agriculture 83:330-337.

Ponchet M, Panabières F, Milat M-L, Mikes V, Montillet J-L, Suty L, … Blein J-P (1999). Are elicitins cryptograms in plant-oomycete communications?. Cellular and Molecular Life Sciences 56:1020-1047.

Qi F, Gong Z-H, Huang W (2006). Study on the selection of cell mutant lines resistant to *Phytophthora capsici* in pepper. Journal of Northwest Sci-Tech University of Agriculture and Forestry (Natural Science Edition) 34:83-88.

Rao S, Ramgoapl S (2010). Effect of *Alternaria helianthi* culture filtrate on callus and regeneration of plantlets from tolerant callus in sunflower (*Helianthus annuus* L.). Indian Journal of Biotechnology 9:187-191.

Rockefeller University (2016). Protocol for silver staining. Proteomics Resource Center. Retrieved 2016 Jan 10 from http://www.proteomics.rockefeller.edu/ms_silverStaining.

Rodpai R, Intapan PM, Thanchomnang T, Sanpool O, Janwan P, Laummaunwai P, … Maleewong W (2016). Strongyloides stercoralis diagnostic polypeptides for human strongyloidiasis and their proteomic analysis. Parasitology Research 115:4007-4012.

Ryan E, Galvin K, O'Connor TP, Maguire AR, O'Brien NM (2007). Phytosterol, squalene, tocopherol content and fatty acid profile of selected seeds, grains, and legumes. Plant Foods for Human Nutrition 62:85-91.

Saubeau G, Gaillard F, Legentil L, Nugier-Chauvin C, Ferrières V, Andrivon D, Val F (2014). Identification of three elicitins and a galactan-based complex polysaccharide from a concentrated culture filtrate of *Phytophthora infestans* efficient against *Pectobacterium atrosepticum*. Molecules 19:15374-15390.

Savita, Virk GS, Nagpal A (2011). *In vitro* selection of calli of *Citrus jambhiri* Lush. for tolerance to culture filtrate of *Phytophthora parasitica* and their regeneration. Physiology and Molecular Biology of Plants 17:41-47.

Sharma A, Wongkham C, Prasongwattana V, Boonnate P, Thanan R, Reungjui S, Cha'on U (2014). Proteomic analysis of kidney in rats chronically exposed to monosodium glutamate. PLoS One 9(12), e116233.

Sopee J, Sangchote S, Stevenson WR (2012). Modified agar-based media for culturing *Phytophthora infestans*. Phytoparasitica 40:269-278.

Souza MM, Oliveira MS, Rocha M, Furlong EB (2010). Antifungal activity evaluation in phenolic extracts from onion, rice bran, and *Chlorella phyrenoidosa*. Ciência e Tecnologia de Alimentos [Food Science and Technology] (Campinas) 30:680-685.

Stong RA, Kolodny E, Kelsey RG, González-Hernández MP, Vivanco JM, Manter DK (2013). Effect of plant sterols and tannins on *Phytophthora ramorum* growth and sporulation. Journal of Chemical Ecology 39:733-743.

Sumbali G (2010). The fungi. Alpha Science International Ltd., Oxford.

Uhlíková H, Obořil M, Klempová J, Šedo O, Zdráhal Z, Kašparovský T, … Lochman J (2016). Elicitin-induced distal systemic resistance in plants is mediated through the protein–protein interactions influenced by selected lysine residues. Frontiers in Plant Science 7:59.

Valencia LDC, Pascual CB, Delfin EF (2014). *In-vitro* selection of pineapple cv. 'Queen' with resistance to culture filtrate of *Phytophthora cinnamomi* Rands. Philippine Journal of Crop Science 39:58-66.

Widmer TL (2014). *Phytophthora palmivora*. Forest Phytophthoras. Retrieved 2017 August 12 from http://dx.doi.org/10.5399/osu/fp.4.1.3557.

Yoder OC (1980). Toxins in pathogenesis. Annual Review of Phytopathology 18:103-129.

Zipfel C, Robatzek S (2010). Pathogen-associated molecular pattern-triggered immunity: veni, vidi…?. Plant Physiology 154:551-554.