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ISSN: 1656-4707
E-ISSN: 2467-5903
Homepage: www.palawanscientist.org

Relative gene expression of calcium binding protein in *Dendrobium bigibbum* Lindl. symbiotically grown with basidiomycetes

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Received: 19 April 2024 || Revised: 12 Nov. 2024 || Accepted: 23 July 2025
Available online: 28 July 2025

How to cite:
Valentino MJG. 2025. Relative gene expression of calcium binding protein in *Dendrobium bigibbum* Lindl. symbiotically grown with basidiomycetes. The Palawan Scientist, 17(2): 52-59. https://doi.org/10.69721/TPS.J.2025.17.2.06

ABSTRACT

Dendrobium bigibbum Lindl. is one of the native species of orchids in the Philippines. Its pharmaceutical potentials have already been explored in Asian countries. Meanwhile, its seed is one of the major constraints for seed germination and mycorrhizal association with compatible basidiomycetes influences the germination of the seeds. In the formation of mycorrhizal association, calcium signaling is one of the initial responses of the plants wherein calcium serves as secondary messenger which triggers physiological functions and changes in the plants and the fungal symbionts as well. In this article, the molecular expression of calcium binding protein was determined in two stages of developments (rhizoid and seedling stage). In vitro cultivation of *D. bigibbum* with selected basidiomycetes were extracted. Quantitative Reverse Transcriptase - Polymerase Chain Reaction was used to determine the Cycle Threshold values. Results revealed the upregulation of the calcium binding proteins which indicates the elevation of calcium concentration, thus, there is a presence of plant-fungal interaction.

Keywords: calcium, orchids, secondary messenger, symbiosis

INTRODUCTION

Orchids is one of the diverse groups of ornamental plants with unique morphological characteristics to attract pollinators forming fungal association for its propagation. Also, most orchids species have medicinal and ornamental properties (Dressler 1993; Cox et al. 1998). *Dendrobium* is among the genus of Orchidaceae with diverse medicinal properties such as against angiogenic, diabetes, cataract, neurological diseases and other disease caused by pathogens (Da Silva and Ng 2017).

Dendrobium is a genus of orchids which can be epiphytic, lithophytic or terrestrial (Nontachaiyapoom et al. 2011). In a study of Bautista and Valentino (2023), the size of *Dendrobium bigibbum* Lindl. embryo ranges from 30.5- 40.25 µm in diameter. Accordingly, for the seed germination to proceed, orchidaceous mycorrhizal association is a necessity (Rasmussen and Rasmussen 2014; Smith and Read 2008).

Bautista and Valentino (2023), revealed that symbiotic association of *D. bigibbum* with the three species of basidiomycetes (*Volvariella* volvacea (Bull.) Singer, *Lentinus tigrinus* (Bull.) Fr., and *Pleurotus florida* Eger), enhanced its germination and growth. The calcium binding proteins responsible for the activation of calcium influx plays important role in the initial establishment of plant fungal symbiotic relationship. The calcium binding proteins are

activated during pre-symbiotic phase which as act marker genes for upstream signaling of calcium are activated and upregulated by fungal in the cortical regions of the plant (Herrbach et al. 2014; Mohanta and Bae 2015). Additionally, they regulate various physiological functions in plant pathogen interaction, plasma membrane mediated responses and plant perceive pathogen/ microbe associated molecular patterns (Luan and Wang 2021).

Transcription factors of calcium binding factors are known to be Ca^{2+}/calmodulin (CaM)-regulated transcription factors and both have a CaM binding domain which are known to mediate plant immunity (Galon et al. 2008). During symbiosis, the Ca^{2+}/CaM-dependent protein kinase (CCaMK) plays an essential role in the interpretation of symbiotic Ca^{2+} signaling in the nucleus for the establishment of symbiotic responses (Gleason et al. 2006; Yuan et al. 2017). Accordingly, calcium acts as a secondary messenger and core regulator during signal transduction following variation in biotic and abiotic factors which can be beneficial or antagonistic in nature cascading physiological and cellular responses (Dodd et al. 2010). The study aimed to determine the expression of calcium-binding proteins in *D. bigibbum* symbiotically grown with three selected fungi.

METHODS

In Vitro Propagation of *D. bigibbum*

Seeds from self-pollinated *D. bigibbum* capsule were used. The capsule was harvested 120 days after visible signs of successful pollination (with enlarged ovary and wilting of flowers). Knudson Orchid Medium (Morel Modification) with modifications (Bautista and Valentino 2023) was used as the growth medium for the in vitro culture of *D. bigibbum*. The seeds were allowed to germinate and develop until rhizoid and seedling stage.

Maintenance of Fungal Inocula

Pure cultures of *V. volvacea, L. tigrinus,* and *P. florida* obtained from the culture collections of Center for Tropical Mushroom Research and Development were grown in a Potato Dextrose Agar (PDA) until the plates were fully colonized with mycelia.

Co-culture Technique

Co-culture technique was carried out by laying filter paper strips in a plate fully colonized with fungal inoculum. The culture plates were then incubated for 30 days at 25 ± 2°C, relative humidity of 23.0% with a photoperiod of 16/8-hours light/dark (Utami and Hariyanto 2019; Chen et al. 2020). For the seedling stage, twenty *D. bigibbum* protocorms with first leaf (90 days after in vitro germination) were selected. These were seeded in plates fully colonized with fungal mycelia. Cultures were incubated for 45 days at 25 ± 2°C with a photoperiod of 16/8-hours light/dark (Chen et al. 2020; Zhang et al. 2020).

Morphological Characterization

The *D. bigibbum* were observed morphologically using dissecting microscope. The plant size was recorded and the appearance of *D. bigibbum* was also noted.

RNA Extraction

Total RNA extraction of *D. bigibbum* during rhizoid and seedling stages were done using the RNAeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. Fifty milligrams (50 mg) of *D. bigibbum* samples were grinded in liquid nitrogen. The powdered tissue was placed in a 2 mL microcentrifuge tubes and 500 µL of RNA lysis buffer was added. It was then vortexed and transferred in a QIAshredder spin column (lilac). The lysate was cleared and adjusted to binding conditions, and was transferred to a RNeasy Mini spin column (pink). Contaminants were eliminated by adding 700 µL RNA wash buffer, 500 µL second RNA wash buffer with Ethanol. Centrifugation was done in each stage at 10,000 rpm for two minutes. After which, the RNA was eluted in 50 µL RNase-free water and placed in a microcentrifuge tube. The extracted RNA was stored at -80°C ultralow freezer (QIAGEN 2024).

Detection of Total RNA

The hardened 1% agarose gel was placed into the gel tank filled with 1×TAE buffer. Three µL of RNA samples were stained with 2x loading dye and were loaded into the agarose gel. The molecular ladder was also loaded in one of the wells of the gel for detection and quantification of the size of the molecule. The power supply was programmed and run at 1-5V/cm between electrodes for 30 minutes. After electrophoresis, the gel was removed from the tray and the excess buffer was drained. It was placed in a computer-controlled gel documentation system. The image was captured and exported into file (Lee et al. 2015).

RNA Quantification

Ribonucleic Acid quantification was performed using photometric nucleic acid quantification using Multi Skan µdrop plate. The µdrop plate was cleaned with sterile distilled water and 2µL of RNA stock solution was placed into the wells of the low-measurement area of the µdrop plate. The plate was loaded into the Multi skan instrument and the template was laid out using the Multi SkanIt software (Thermo Fisher Scientific Corporation 2015).

The absorbance at 260 nm was recorded and the amount of RNA was calculated using the formula:

RNA concentration (µg/mL) = Abs260 × 40 µg/mL × 20

Based from the computation the following concentration of RNA obtained were 461.952 µg/mL (*D. bigibbum* with *V. volvacea*), 218.688 µg/mL (*D. bigibbum* with *L. tigrinus*), 348.48 µg/mL (*D. bigibbum* with *P. florida*) and 310.248 µg/mL (asymbiotically grown *D. bigibbum*)

Gene Expression Analysis

ViPrimePLUS One Step Taq RT-qPCR Green Master Mix I was used for qrt-PCR following the manufacturer's guide using the Step One instrument (Applied Biosystem, USAEach reaction contained 5µL Taq One Step RT-qPCR Green Master Mix I, 1µL primer/probe mix, 2.5µL RNA (25ng), and 2µl nuclease free water. The primer used for calcium binding protein (Forward-GTCGTTGGAGAATACGAAGAG; Reverse-TGTGCGTCGTGAGATCCAGATAATG) while EF-1α (Forward-TGTGGTCCCAATCATCCC; Reverse-ATAGGCTTGCTGGGAACC) served as the housekeeping gene. The 10 µL of prepared master mix was loaded into a 0.2 PCR multiwell plate and sealing film was used to cover the multi-well plate to prevent evaporation when heated. The multi-well plate was loaded in the Step One Instrument RT-PCR platform and was set up based from the optimized standard cycling conditions EF-1α served as the housekeeping 36 gene to normalize transcript abundance. Each RT-qPCR assay was replicated thrice. Finally, the relative gene expression was calculated using the Livak method or the conventional 2 −ΔΔCT method (Livak and Schmittgen 2001; Chen et al. 2020). Livak method for relative gene expression analysis assumes 100% and within 5% efficiencies of gene amplification of the target and reference genes. The Cycle threshold (Ct values) of the target and the reference genes were normalized using the formula $\Delta Ct_{(test)} = Ct_{(target,\ test)} - Ct_{(ref,\ test)}$. Then, the ΔCq was normalized using the formula $\Delta\Delta Ct = \Delta Ct_{(test)} - \Delta Ct_{(calibrator).}$ Finally, the normalized gene expression was computed using the formula 2-ΔΔCt. Expression of gene is said to be upregulated when the computed fold change is statistically significantly higher as compared to the reference genes.

Statistical Analysis

The study was laid out using completely randomized design. Test for difference was done using Analysis of Variance (ANOVA) and comparison among means by post-hoc test of homogenous subsets through Tukey's HSD test for the plant size and t-test for the expression of calcium binding protein. Significant level of difference was set at 0.05 level of significance.

RESULTS

The expression of calcium binding protein (DoCML19- Dendrobium_GLEAN_10016982) was analyzed in rhizoid and seedling stages of *D. bigibbum* symbiotically grown with the selected basidiomycetes. During early stages of seed germination, the protocorm formation indicating the symbiotic association was observed. Additionally, protocorm responds to the fungal hyphae that enters the embryo at the basal end. As seen in Figure 1(D, E) via Scanning Electron Microscope, mycelial colonization is evident on the surface of the roots of the *D. bigibbum* seedlings and on the protocorm during the rhizoid stage.

In addition, morphological assessment of *D. bigibbum* in terms of plant size also revealed that *D. bigibbum* co-cultured with the selected fungi were significantly higher as compared to asymbiotically grown *D. bigibbum* both in rhizoid and seedling stages (Table 1). Morphologically and symbiotically grown *D. bigibbum* are vibrant green in color, intact leaf and roots (Figure 1). Meanwhile, as shown in Figure 2, asymbiotically grown *D. bigibbum* are dull green in color, and the lower parts of the protocorm is colored brown which is an indication of oxidation and early senescence of *D. bigibbum*. These coincide with the expression of calcium binding proteins. Presented in Figure 3 and Table 2 is the relative gene expression of calcium binding protein in *D. bigibbum.* In rhizoid stage, the expression of gene in terms of fold change was significant higher when co-cultured with *V. volvacea* (5.20), *L. tigrinus* (3.93) and *P. florida* (1.67) as compared to the asymbiotically cultured *D. bigibbum* (1.01). Similarly in seedling stage, upregulation of gene was recorded with 40.34, 28.70- and 5.83-fold change in *V. volvacea, P. florida* and *L. tigrinus,* respectively. Based from the statistical analysis, in both rhizoid and seedling stage, the calcium binding protein was upregulated when grown symbiotically as compared to asymbiotically grown *D. bigibbum*.

Upregulation of calcium-binding protein was observed in all *D. bigibbum* co-cultured with basidiomycetes both in rhizoid and seedling stage, however, relative expression is noticeably higher during seedling stage.

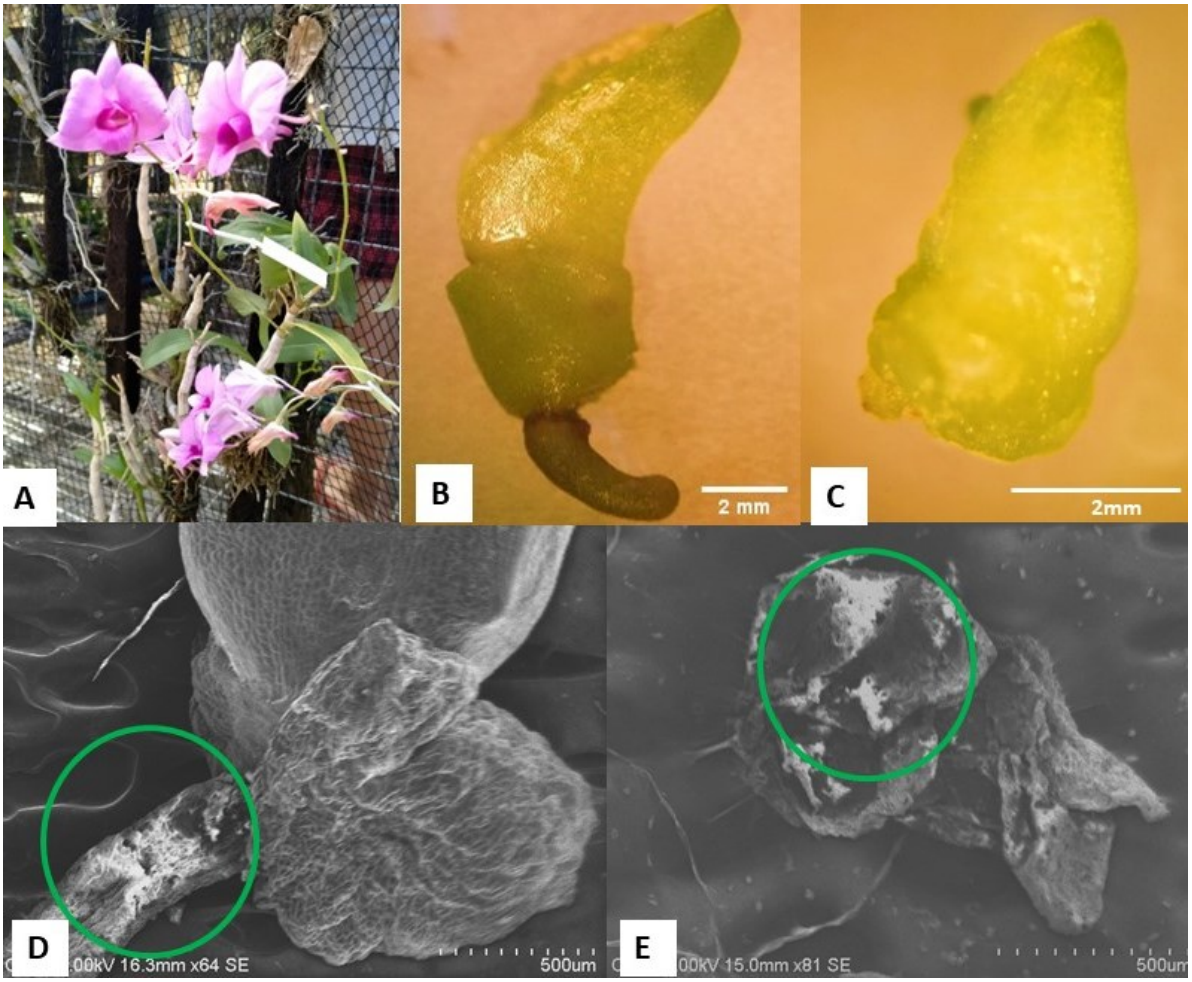

Figure 1. The *Dendrobium bigibbum.* A) *in situ;* B) seedling stage under dissecting microscope; C) rhizoid stage under dissecting microscope; D) seedling with fungal colonization on the root under scanning electron microscope; E) rhizoid stage with fungal colonization at the protocorm under scanning electron microscope.

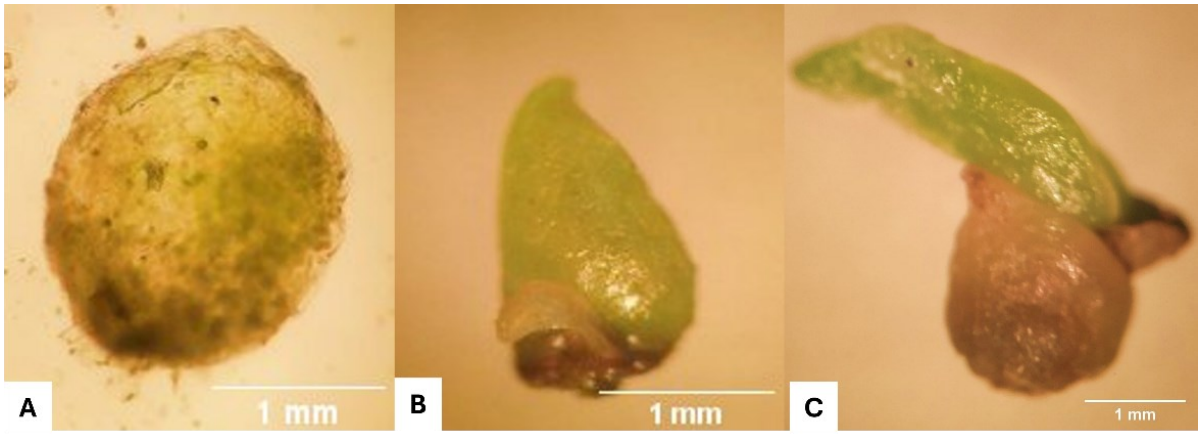

Figure 2. Asymbiotically grown *Dendrobium bigibbum.* A) germinated protocorm; B) rhizoid stage under dissecting microscope; C) seedling stage under dissecting microscope.

Table 1. Plant size of *Dendrobium bigibbum* in rhizoid and seedling stage (mm). *Values are the Mean ± SD. Means within a column having the same letter of superscript are insignificantly different from each other at 0.05 level of significance using Tukey's test.

Treatments	Rhizoid stage	Seedling stage
D. bigibbum co-cultured with *V. volvacea*	7.22 ± 0. 932[a]	18.59 ± 0.893[a]
D. bigibbum co-cultured with *L. tigrinus*	4.35 ± 0.667[b]	16.23.74 ± 0.594[a]
D. bigibbum co-cultured with *P. florida*	5.13 ± 0.265[bc]	14.32 ± 0.890[ab]
Asymbiotically cultured in PDA	3.83 ± 0.989[c]	12.86 ± 0.574[b]

Table 2. Expression level of DOCML19 (calcium-binding protein) in rhizoid and seedling stage of *Dendrobium bigibbum.* *Values are the Mean ± SD. Means within a column having ** are highly significant and * are significantly different from the reference gene at 0.05 level of significance; ↑ indicates upregulation of the DOCML19 gene.

Treatments	Rhizoid Stage	Seedling Stage
D. bigibbum co-cultured with V. *volvacea*	5.20 ± 0.120479**↑	40.34±11.38749**↑
D. bigibbum co-cultured with *L. tigrinus*	3.93 ± 0.414109**↑	5.83±2.685932*↑
D. bigibbum co-cultured with *P. florida*	1.670.24547* ↑	28.76 ± 6.52658**↑
Asymbiotically grown *D. bigibbum*	1.01 ± 0.3509	1.030 ± 0.7841

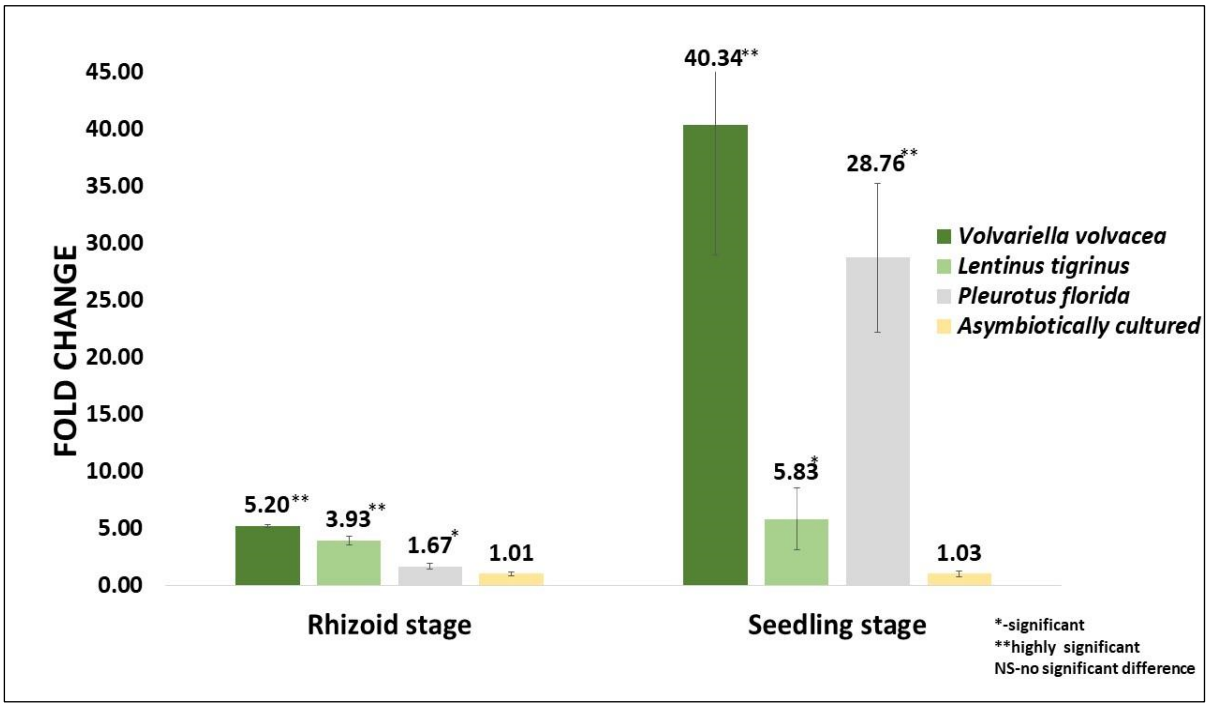

Figure 3. Expression level of DOCML19 (calcium-binding protein) in rhizoid and seedling stage of *Dendrobium bigibbum.*

DISCUSSION

There are different stages for the formation of mycorrhizal association of orchids and fungi, such as the attraction of the symbiont, initial contact, initial fungal colonization, proliferation of the fungal hyphae within the orchid tissues, and colonization of the cortical cells. Mycorrhizal symbiosis produces signaling molecules, such as strigolactones from plant roots, attracting fungi and stimulating hyphal branching (Akiyama et al. 2005; Kretzschmar et al. 2012; Valadares et al. 2012). Initial contact takes place when a compatible symbiont accesses the plant tissues with the formation of appressorium prior to hyphal penetration and fungal entry to the protocorm via the suspensor which occurs enzymatically (Smith and Read 2008). Lastly, the cortical cells will be colonized by the symbiont; this involves invagination of plant

plasma membrane, cytoskeletal rearrangements, nuclear change, other organelles change, peloton formation, and interfacial matrix formation (Perotto et al. 2014).

During this time, the change in cytoskeleton causes cortical microtubule depolarization, nuclear movement, and realignment to the peleton hyphae (Genre et al. 2005). Also, cytoplasmic changes trigger the breakdown of starch leading to metabolites' extensive membrane remodeling in colonized cells for plant metabolism and defense via the production of enzymes, such as polyphenol oxidase, peroxidase, and catalase, which are known as antioxidants (Valadares et al. 2012). Meanwhile, peloton functions to provide the nutrient transfer area between the symbionts where there is a high polyphenol oxidase activity for the breaking down of phytoalexins. There will then be a production of interfacial matrix by the orchids which is marked by the formation of callose, cellulose, and pectin around the collapsing pelotons, followed by septal disintegration. During these events, calcium act as secondary messenger (Bonfante 2001; Armstrong and Peterson 2002; Yi and Valent 2013).

Based from the studies of Dearnaley et al. (2007) and Chen et al. (2020), upregulation of calcium binding protein depicts the increase of cytosolic calcium which is an initial sign of infection or the host plant can be challenged by mutualistic or pathogenic partners. Accordingly, increase in calcium plays vital role in plant immunity by regulating the Salicylic acid biosynthesis via the positive regulation of isochorismate synthase 1(ICS1) and enhanced disease susceptibility 1(EDS1) (Dodd et al. 2010; Kudla et al. 2010; Seybold et al. 2014; Tsuda and Somssich 2015). Transcription factors of calcium binding factors are known to be Ca^{2+}/calmodulin (CaM)- regulated transcription factors and both have a CaM binding domain which are known to mediate plant immunity (Galon et al. 2008; Kim et al. 2009; Wang et al. 2009; Zhang et al. 2010; Reddy et al. 2011; Bickerton and Pittman 2012).

During symbiosis, the Ca^{2+}/CaM-dependent protein kinase (CCaMK) function in initiation of symbiotic responses by Ca^{2+} signaling in the nucleus (Gleason et al. 2006; Yuan et al. 2017). In the process, Nod A factor which is responsible for the nodulation, activates the calcium binding proteins which in return induces the nodulation (Gleason et al. 2006). Similarly, the increment in calcium concentration can be attributed to the formation of root symbiosis, where the secondary metabolites, flavonoids such as luteolin and naringenin are being release for the biosynthesis of Nod factor (Moscatiello et al. 2010; Cui et al. 2019). Additionally, Reid et al. (2016, 2017) suggested that pre-interaction activity in symbiotic association, root-nodule symbiosis and cytokinin biosynthesis is regulated by Ca^{2+} signaling.

According to Wang et al. (2020), calcium is regulated by GTPases during the process of symbiosis. Wherein, the calcium channels are activated the radical oxygen species that cascades calcium influx for plant immune responses. Moreover, as soon as interaction and recognition between the host plants and the microorganisms occur the intracellular calcium levels increase and minimal change in the calcium concentration could greatly affect the physiological activity and interaction of organisms (Harper and Harmon 2005; Charpentier et al. 2008; Mazars et al. 2009). Once the calcium level increased, it cannot be synthesized nor degraded thus the efflux and the influxes' reaction must maintain homeostasis (Lecourieux et al. 2002).

Meanwhile the difference on the concentration of calcium binding protein in seedling and rhizoid stage can be attributed to the specificity of calcium responses. Moreover, different species of orchids may interact with mycorrhizal association at different phases, some during the early phase of germination while others during the protocorm to adult phase of development (Rasmussen and Rasmussen 2014). Fungi that contribute to the development of orchids include the *Rhizoctonia*-like fungi complex, wood or litter-decomposing fungi, and fungal endophytes (Da Silva et al. 2015; Herrera et al. 2017). They serve as sources of minerals and nutrients in partially mycoheterotrophic orchids until the first green leaf emerges, while autotrophic orchids are less dependent on mycorrhizal association once the roots and leaves are formed (Herrera et al. 2017). Also, upon the host penetration of fungal symbiont, biosynthesis of hormones by the host plant in the infected tissues and the production of hormones by the infecting fungi may also take place, which aids in the development of the host (Robert-Seilaniantz et al. 2007). Fungal symbionts may have little effect on seed germination but may significantly increase the growth of orchids during the seedling stage. However, different fungal symbionts have also different effects on growth and development which can be attributed mainly due to development-dependent specificity (Zhang et al. 2020).

FUNDING

The research was initiated and funded by the author.

ETHICAL CONSIDERATIONS

The study is exempted from any ethics guidelines. Experimental animals are not involved in the study.

DECLARATION OF COMPETING INTEREST

The author declares no conflict of interest.

ACKNOWLEDGMENTS

The author would like to acknowledge the Central Luzon State University, the anonymous reviewers and editors of The Palawan Scientist.

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Responsible Editor: Alangelico O. San Pascual, MSc