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## Expression of genes associated with the growth of symbiotically grown *Dendrobium bigibbum* Lindl.

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**Abstract** The study evaluated the expression of genes related to the growth of symbiotically grown *Dendrobium bigibbum* were evaluated (biosynthesis/ signal transduction of auxin and gibberellins, common symbiotic pathway, and mycorrhizal-induced putative genes). Results revealed that the relative expression of genes were upregulated in *D. bigibbum* co-cultured with *Volvariella volvacea*. Meanwhile, in *Lentinus tigrinus*, DOSAUR71 (seedling stage) was downregulated and the cycle threshold value for DoSWEET14 was unchanged. In *D. bigibbum* co-cultured with *Pleurotus florida*, downregulation of GA3ox (seedling stage), DOSAUR71 (rhizoid and seedling stages) and DoIPM (rhizoid stage) were observed and unchanged relative gene expressions of DoIPM (seedling stage) and DoSWEET14 (seedling stage) were recorded. Thus, both *V. volvacea* and *L. tigrinus* formed compatible mycorrhizal association with *D. bigibbum* which caused increase in the growth of *D. bigibbum* during rhizoid and seedling stages.

**Keywords:** CT value, Gene expression, Orchidaceous mycorrhiza, Rhizoid, Seedling

### Introduction

Orchidaceous mycorrhizal association correspond to symbiotic associations between fungal partner and orchid species, wherein the fungi provide the sources of carbon, nitrogen, phosphorus, and other elements (Brundrett, 2002; Cameron *et al.*, 2006; Waterman *et al.*, 2009). According to Taylor *et al.* (2004), interactions provide diversity and adaptive evolution. The process starts with the hyphal penetration and infection process which may occur within the epidermal hair, suspensor cells, rhizoids, or rhizomes. Then, establishment of symbiosis cause the peloton formation and lysis, followed by nutrient transfer (Rasmussen and Whigham, 2002; Steinfort *et al.*, 2010). Fungal pathogens, endophytes, and saprophytes are included as mycorrhizal fungi of orchids (Rasmussen, 1995;

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Brundrett, 2002; Taylor *et al.*, 2004; Steinfort *et al.*, 2010). Several studies had already revealed the effect of compatible fungi in growth and development of orchids. In the recent study of Bautista and Valentino (2023), the compatible symbiotic association of three basidiomycetes resulted to increment in the growth of *Dendrobium bigibbum*.

Molecular analysis of genes for growth and development of *D. bigibbum* such as genes which are involved in biosynthesis/ signal transduction of auxin and gibberellins, common symbiotic pathway, and mycorrhizal-induced putative genes were evaluated. Based on the study of Chen *et al.* (2020; 2022), these genes are considered as differentially expressed genes in orchids which are presented during seed germination and seedling stage.

The study aimed to evaluate the expression of genes associated with the growth of symbiotically grown *D. bigibbum*.

## **Materials and methods**

### ***Preparation of culture media***

Germination of *D. bigibbum* seeds were carried out using Knudson Orchid Medium (Morel Modification) with modifications as described in the study of Bautista and Valentino (2023). Meanwhile, the pure culture of basidiomycetes (*Volvariella volvacea*, *Lentinus tigrinus* and *Pleurotus florida*) were cultivated in Potato Dextrose Agar.

### ***In- vitro co-culture of D. bigibbum and selected basidiomycetes***

The three fungal isolates namely *Volvariella volvacea*, *Lentinus tigrinus* and *Pleurotus florida* (were grown in PDA for seven days. Filter paper strips were then placed in the surface of the fully ramified plates. Germinated *D. bigibbum* under rhizoid and seedling stage were selected and transferred in the filter paper strips. The cultures were incubated for 30days (for rhizoid) and 45 days (for seedling) at  $25\pm 2^{\circ}\text{C}$  with a photoperiod of 16/8-hours light/dark (Utami and Hariyanto, 2019; Chen *et al.*, 2020 as cited by Bautista and Valentino, 2023).

### ***Molecular analysis of genes involved in growth and development of D. bigibbum***

Molecular analysis was done to determine the expression of various genes involved in the growth and development of symbiotically grown *D. bigibbum*. These include the genes for gibberellic acid biosynthesis GA3ox (gibberellin 3-beta-dioxygenase) and SCL3 (scarecrow-like 3 protein-DELLA protein); genes

involved in indole acetic acid biosynthesis and signaling DoIPM (YUCCA family monooxygenase), and DOSAUR71 (SAUR family protein); genes for common symbiotic pathway (DoNSP2 (nodulation-signaling pathway 2 protein) and DOSWEET14 (bidirectional sugar transporter SWEET14-like), and mycorrhizal putative genes which include DOHAL (chitinase), DoCRI (fatty acid desaturase), and DoGLU (beta-1,3-glucanase). 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.

### ***Detection and quantification of total RNA***

The quality of the extracted RNA was determined through gel electrophoresis and was viewed through a computer-controlled gel documentation system. Meanwhile, Multi Skan  $\mu$ drop plate was used for photometric nucleic acid quantification. The absorbance at 260 nm was recorded and the amount of RNA was calculated by using the formula:

$$\text{RNA concentration } (\mu\text{g/ml}) = \text{Abs}_{260} \times 40 \mu\text{g/ml} \times 20$$

### ***Gene expression analysis***

ViPrimePLUS One Step Taq RT-qPCR Green Master Mix I was used following the manufacturer's guide using the Step One instrument (Applied Biosystem, USA). The primers used for the qRT-PCR analysis were designed specifically for each target genes. The relative gene expression was calculated using the Livak method (Chen *et al.*, 2020; Livak and Schmittgen, 2001).

## **Results**

Relative expression of genes involved in the growth and development of *D. bigibbum* grown symbiotically with basidiomycetes (*Volvariella volvacea*, *Lentinus tigrinus* and *Pleurotus florida*) were determined during two stages of development, rhizoid and seedling stage. The summary of the relative expression of the target genes is shown in Table 1. When *D. bigibbum* was co-cultured with *V. volvacea*, all evaluated genes were upregulated, whereas when grown with *L. tigrinus*, DoSAUR71 was downregulated, and unchanged relative gene expression was recorded in DoSWEET14 during seedling stage. In *D. bigibbum* co-cultured with *P. florida*, GA3ox, DoIPM were downregulated in seedling stage; while unchanged relative expression of genes was recorded in DoSAUR71 (rhizoid and seedling stages) and DoSWEET14 during seedling stages. The stages of growth of *D. bigibbum* from flower to seedling is presented in Figure 1.

**Table 1.** Summary of the relative expression of genes associated with growth and development of *D. bigibbum* co-cultured with selected basidiomycetes

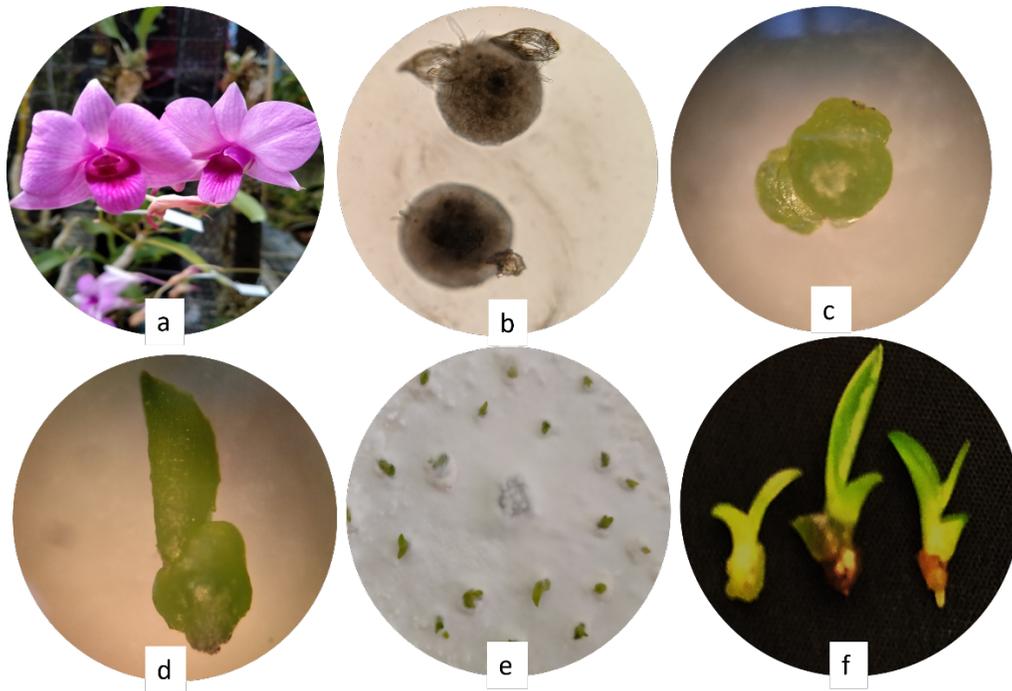
Genes associated with growth and development of <i>D. bigibbum</i>	RHIZOID			SEEDLING		
	<i>D. bigibbum</i> co-cultured with					
	<i>V. volvacea</i>	<i>L. tigrinus</i>	<i>P. florida</i>	<i>V. volvacea</i>	<i>L. tigrinus</i>	<i>P. florida</i>
GA3OX (gibberellin 3-beta-dioxygenase)	17.32±0.87↑	10.77±4.58↑	5.80±0.51↑	48.72±31.45↑	7.84±1.650↑	0.21±0.12↓
SCL3(Scarecrow-like 3 protein)	7.78±2.00↑	6.66±0.64↑	5.00±2.33↑	86.62±10.81↑	26.11±9.01↑	4.12±0.83↑
DoIPM( YUCCA family monooxygenase)	8.06±1.87↑	4.31±0.90↑	2.43±0.80 NC	6.86±1.44↑	2.62±0.295↑	0.29±0.12↓
DoSAUR71 (SAUR family protein)	2.74±0.59↑	1.44±0.18↑	0.98±0.022 NC	3.24±0.08↑	0.70±0.04↓	1.05±0.029 NC
DoNSP2 (nodulation-signaling pathway 2 protein)	39.47±19.0↑	7.92±0.42↑	1.47±0.10↑	14.10±1.45↑	4.03±0.43↑	3.65±1.11↑
DoSWEET14(bidirectional sugar transporter SWEET14-like)	8.76±1.16↑	6.49±2.78↑	4.51±0.54↑	3.17±0.94↑	1.71±0.07NC	1.94±0.48 NC
DoHAL (chitinase)	2.82±1.27↑	1.47±0.08↑	4.22±0.78↑	24.52±4.63↑	2.55±1.92↑	14.94±1.56↑
DoCRI (fatty acid desaturase)	103.87±67.1↑	5.65±0.54↑	5.06±0.95↑	66.35±5.55↑	5.29±0.66↑	1.34±0.44↑
DoGLU (beta 1,3-glucanase)	1.51±0.20↑	19.15±5.53↑	2.14±0.26↑	12.86±4.78↑	8.67±2.50↑	18.45±1.20↑

↑ upregulated ; ↓ downregulated ; NC- no change

For the signaling and biosynthesis of gibberellic acid, gibberellin 3-beta-dioxygenase (GA3ox) and scarecrow-like 3 proteins (SCL3) were evaluated (Figure 2.a and b). Results showed the upregulation of GA3ox in *D. bigibbum* co-cultured with the three basidiomycetes with the highest fold change in *D. bigibbum* co-cultured with *V. volvacea* (17.32, 48.72), *L. tigrinus* (0.77, 7.84) in both seedling stage and rhizoid stage, respectively. For those grown with *P. florida*, upregulation to downregulation of GA3ox from the rhizoid stage (5.80) to seedling stage (0.21) was detected. Whereas, SCL3 was upregulated when *D. bigibbum* was symbiotically grown with basidiomycetes both during rhizoid and seedling stage.

Meanwhile, two genes for the biosynthesis of indole acetic acid namely DoIPM (YUCCA family monoenase) and DoSAUR71(SAUR family protein) were observed. Significant increase in the relative gene expression of YUCCA during rhizoid and seedling stage were observed when *D. bigibbum* was co-cultured with *V. volvacea* and *L. tigrinus* while it was downregulated in *D. bigibbum* co-cultured with *P. florida*. It was also noticeable that the relative gene expression in the rhizoid stage was higher compared to the seedling stage (Figure 2.c). As depicted in Figure 2.d, for the SAUR family protein, upregulation of gene expression was observed during the rhizoid stage when *D. bigibbum* was co-cultured with *V. volvacea* (2.74) and *L. tigrinus* (1.44). During the seedling stage,

the gene was upregulated when co-cultured with *V. volvacea* (3.24), downregulated when co-cultured with *L. tigrinus* (0.70).

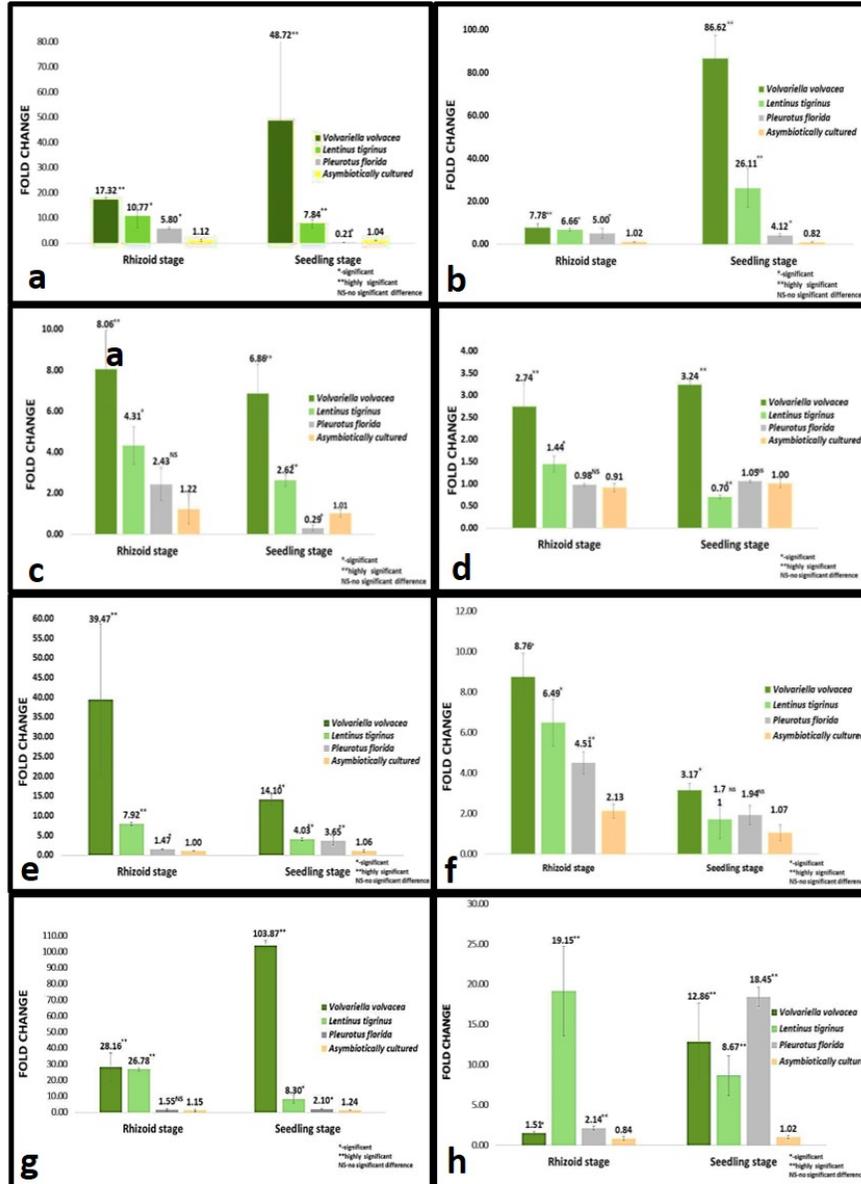


**Figure 1.** *Dendrobium biggibum* a. flower b. germinating seeds c. protocorm stage d. rhizoid stage e. co-culture with basidiomycetes f. seedlings

DoNSP2 (nodulation-signaling pathway 2 protein) and DOSWEET14 (bidirectional sugar transported SWEET14-like) were assessed for the common symbiotic pathway (Figure 2. g, h, i). Upregulation in the relative expression of DoNSP2 was higher during the rhizoid stage as compared to the seedling stage when co-cultured with *V. volvacea* (14.10,39.47) and *L. tigrinus* (4.03,7.92), while when co-cultured with *P. florida* (3.65, 1.47), the expression of DoNSP2 was higher in the seedling stage. For the relative expression of the DOSWEET14 in the rhizoid stage ranging from 8.76 to 4.51-fold change, upregulation of gene was shown in all *D. biggibum* co-cultured with the three basidiomycetes, while in the seedling stage, only *V. volvacea* caused the upregulation of DoSWEET14 recorded.

For the mycorrhizal putative genes, three genes such as DoHAL, DoCRI, and the DoGLU were evaluated. Based from the study of Chen *et al.* (2022), the aforementioned genes are upregulated in symbiotic germination of orchids from early germination to seedling formation. According to Zhao *et al.* (2013), the production of chitinase and beta-1-3, glucanase in plants is a response to infections and induced by fungi necessary for the lysis of the fungal cell wall. In addition, combination of beta-1-3, glucanase, and chitinase protect the plants through direct decomposition of fungal cell wall and indirectly via the release of elicitors. For the relative expression of DoHAL (Figure 2h.), co-culture with three basidiomycetes caused its upregulation during seedling stage, with the highest fold change in *V. volvacea* (24.52). During seedling stage, upregulation was recorded when co-cultured with *V. volvacea* and *P. florida*. For the relative expression of fatty acid desaturase (DoCRI), the gene was upregulated when *D. bigibbum* was co-cultured with *V. volvacea* and *L. tigrinus* while during the seedling stage, it was recorded in all *D. bigibbum* seedlings symbiotically grown with the three basidiomycetes. Lastly, DoGLU was upregulated in both stages when co-cultured with the three basidiomycetes. In the rhizoid stage, the highest gene expression was recorded when co-cultured with *L. tigrinus* (19.15), while during the seedling stage *D. bigibbum* co-cultured with *P. florida* (18.45) registered the highest relative gene expression.

Overall, relative expression of genes involved for the growth and development of the *D. bigibbum* were changed when grown symbiotically with *V. volvacea*, *L. tigrinus*, and *P. florida*. The upregulation of all genes analyzed can be reflected on the outstanding growth of *D. bigibbum* co-cultured with *V. volvacea*. Majority of genes were upregulated when co-cultured with *L. tigrinus* except for the downregulation of DOSAUR71 and unchanged expression of DoSWEET14. Lastly, negative and statistically non-significant effect of *P. florida* occurred due to downregulation of DoSAUR71 (rhizoid and seedling) and unchanged relative gene expressions of GA3ox and DoSWEET14.



**Figure 2.** Relative expression of genes associated with the growth of *D. biggibum* a. GA3OX (gibberellin 3-beta-dioxygenase) b. SCL3(DELLA protein) c. DOIPM (YUCCA family monooxygenase) d. DASUR71 (SAUR family protein) e. DONSP2 (nodulation-signaling pathway 2 protein) f. DOSWEET14 (bidirectional sugar stage transporter SWEET14-like) g. DoHAL(chitinase) h. DOCRI (fatty acid desaturase) i. DogLU ((beta 1,3-glucanase)

## Discussion

Various genes associated with the growth and development of *D. bigibbum* were assessed to determine the symbiotic effect of the three selected basidiomycetes. As revealed in the results, orchids-mycorrhizal association significantly affected the upregulation of the evaluated genes.

The upregulation of GA-related genes caused increment in the growth rate of *D. bigibbum*. This is due to the catalytic action of GA3ox, which catalyzes the conversion of GA20 to biologically active GA1 and upregulation of GA3ox also increases the amount of GA in tissue-specific and development-specific manner (Reinecke *et al.*, 2013; He *et al.*, 2019). GA3ox is expressed in cortex and endodermis of the germinating seeds and will diverge in the later stages of growth in plant (Yamaguchi *et al.*, 1998). In a study of Yin *et al.* (2022), increasing the amount of GA promotes elongation of the 1<sup>st</sup> and the 2<sup>nd</sup> leaves of *Paphiopedilum callosum*. Meanwhile, SCL3 directly promotes bioactive GA. In a study of Zhang *et al.* (2011), an elevated expression of GA biosynthesis genes was detected in the germinating seeds, seedling, and seedling roots which indicates that SCL3 functions as a positive regulator of GA signaling and plays a crucial role during germination and seedling development. Additionally, Heo *et al.* (2011), showed that the endodermis-expressed SCL3 mediates GA-promoted cell elongation in the root.

For the auxin related genes, based from the studies of Zhao *et al.* (2013) and Cao *et al.* (2019), YUCCA genes regulate the production of auxin during plant-microbes and plant-plant interactions through the catalysis of oxidative decarboxylation of the indole 3-pyruvic acid forming the indole 3 acetic acid and are present in the. Additionally, SAUR is one the largest family of early auxin response genes that are specific to plants which are involved in cellular expansion through cell wall acidification during seedling stage (Stamm and Kumar, 2013; Spartz *et al.*, 2014). They function in regulating leaf initiation, lamina formation and vein development. Over expression of SAUR also lead to the increase transport of auxin due to the increase in plasma membrane proton ion pump (Ren and Gray, 2015).

In relation to *D. bigibbum* symbiotically cultured with basidiomycetes, noticeable increase in the growth of *D. bigibbum* were observed when co-cultured with *V. volvacea* (both in rhizoid and seedling stages) and *L. tigrinus* (rhizoid stage). Symbiotic association resulted to the expression of genes related to endogenous auxin which could lead to faster differentiation of the embryo and promotes protocorm stage (Chen *et al.*, 2020). In addition, shoot formation during orchids germination is due to the establishment of appropriate auxin gradients (Novak *et al.*, 2014). Also, Barker and Tagu (2000), Meixner *et al.* (2005) and

Chanlud and Morel (2016), mentioned that plants interacting with mycorrhizal fungi contains high amount of auxin which can act in the formation of symbiotic interaction, nodule formation and host invasion.

NSP2 is also essential for the CCaMK-activated cytokinin signaling pathway via the cytokinin receptor CRE1 leading to cortical cell division and nodule organogenesis (Tirichine *et al.*, 2007; Ariel *et al.*, 2012). SWEET genes which are found in the plasma membrane functions as sucrose, glucose, and GA transporters. Once they gained GA transport activities, these proteins assumed important roles in plant growth, which could affect seed development, early seedling growth and anther development (Kanno *et al.*, 2016).

The results on the expression of SWEET14 genes when co-cultured with basidiomycetes coincide with the study of Chen *et al.* (2020), wherein it is highly expressed in the presence of mycorrhizal symbiosis. Based on the result of the present study, it is highly expressed during the rhizoid stage in *D. bigibbum* co-cultured with basidiomycetes but the unchanged expression of genes during the seedling stage except those with *V. volvacea* was observed. Thus, this can be one of the factors for the high increase in growth of *D. bigibbum* co-cultured with *V. volvacea* in terms of length of leaf and fresh weight. SWEET genes are induced by mycorrhizal association, wherein upregulation of SWEET genes is associated with the increased capacity of the fungal symbionts to sustain the adequate sugar metabolism and mobilization for plant growth (Perotto *et al.*, 2014; Breia *et al.*, 2021).

Beta-1,3-Glucanases are abundant in plants and function in cell division, trafficking of materials through plasmodesmata, abiotic and biotic stress defense, and formation of seed. In addition, they are known to combat fungal pathogens in association with chitinases and antifungal proteins (Balasubramanian *et al.*, 2012). Fukamizo and Shinya (2019), mentioned that chitinase enhances plant defense by acting on the chitin of the fungi and also enhances plant growth and yield. It is present during symbiosis and nodule development. In terms of plant growth and development, it is involved in the regulation of organogenesis and plays important role in germination through degradation of chitooligosaccharides in the cell walls of seed coat. Results were in agreement with the studies of Zhao *et al.* (2013) and Perotto *et al.*, (2014), wherein increase in chitinase expression was found in symbiotic protocorms. In case of fatty acid desaturase, Dar *et al.* (2017) found out that in mycorrhizal symbiosis, fungi increased their lipid content which are transferred from the plants. Increase in FAD is necessary for signaling molecules and plant growth regulators (Cowan, 2006). Lastly beta-1,3-glucanase has evolved several mechanisms to recognize microbial infections and respond adequately by activating defense responses. In combination with chitinase, they hydrolyze the cell wall of the fungal pathogen wherein the b-1,3-glucanase

degrades the glucan while the chitinase will attack the bond of chitins in the fungal cell walls (Mohammadi and Karr, 2002). In addition, in a study of Chen *et al.* (2020), the expression of the glucanase gene can be affected by the concentration of GA present, and a high expression can be observed during interaction to limit the entry of fungal hyphae to the host plant.

Overall, co-culture of *D. bigibbum* with basidiomycetes (*P. florida*, *L. tigrinus* and *V. volvacea*) resulted to changed in the expression of genes associated to the growth of in-vitro grown *D. bigibbum*.

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