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Research Article
Seed Viability and Symbiotic Seed Germination in *Vanilla* spp. (Orchidaceae)

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Abstract

Background: Mycorrhizal interactions are ecologically important to understand the orchid biology since orchids depend on mycorrhizal fungi for seed germination. **Objective:** The aim of this study was to evaluate the ability of fungi isolated from the roots of *Vanilla* adult plants to induce symbiotic seed germination. **Materials and Methods:** Fruits from three native species of *Vanilla* from Colombia were collected to test symbiotic seed germination through experiments *in vitro*. First, the viability of the seeds was determined through scarification of the seeds using sand-paper and immersion of the embryos in a 1% tetrazolium chloride solution. Second, a surface-sterilized seeds suspension was added to the petri dishes that had an isolated fungus previously obtained from adult plants. Finally, the percentage of seed germination and the index of growth were calculated and the germination stages were morphologically characterized. A one-way analysis of variance (ANOVA) for evaluate differences among species was realized for seed viability variable. For seed germination trails, a factorial design was used and a generalized linear model with a gamma distribution and link function "Log" was performed. **Results:** With *Ceratobasidium* strains percentages of germination close to 80% were reached whereas, *Tulasnella* isolates promoted percentages close to 60%. At *Vanilla* species level, *V. rivasii* had the best performance for the variables evaluated. **Conclusion:** This study presented a new procedure for measuring *Vanilla* seed viability, as well as, it provided relevant information about seed development. Both *Ceratobasidium* and *Tulasnella* isolates can promote germination in *Vanilla* seeds. However, with the *Ceratobasidium* strains, higher stages of development were reached.

Key words: Orchids, *Ceratobasidium*, *Tulasnella*, mycorrhizal fungi, seed development, tetrazolium, *Vanilla*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The genus *Vanilla* Plumier ex Miller (Orchidaceae) is economically important because some of its species are used to extract vanillin for food and perfume industry purposes. Vanillin is one of the most economically important spice in the world[1]. The most cultivated species in the genus is *V. planifolia* Andrews and the countries with highest vanilla production in the world were Madagascar, Reunion Island, French Polynesia and Indonesia[2,3]. However, these countries have been withdrawing from the business of vanillin due to the pathogenic fungus *Fusarium oxysporum* f. sp. *radicis-vanillae*, which attacks severely the crops[3]. In Colombia, *Vanilla* genus is distributed below 2000 m in elevation (m.a.s.l.) with 22 reported species[4-8].

Like the seeds of other orchid species, *Vanilla* species produce many small seeds without endosperm and low germination rate under natural conditions[9-11]. The low germination is attributed to the lack of an appropriated mycorrhizal fungus that provides essential nutrients for embryo development[12-17]. Usually, these Orchid Mycorrhizal Fungi (OMF) belongs to Rhizoctonia-like fungi[18-22] which include the teleomorph genera *Ceratobasidium*, *Tulasnella*, *Sebacina* and *Thanatephorus*[23,24].

Previous research on *Vanilla* mycorrhizal interactions included four species from Puerto Rico, Costa Rica and Cuba, where they found differences between aerial and terrestrial roots[25]. The most frequent terrestrial root fungi was *Ceratobasidium* while aerial roots were more associated with *Tulasnella*. In the germination test, only 7% of the seeds germinated with *Ceratobasidium* strains but there was no germination with *Tulasnella* isolates. In Colombia, one study reported endophytic fungi in *Vanilla* species but germination was no evaluated[26]. Mosquera-Espinosa *et al.*[27] reported a *Ceratobasidium* as mycorrhizal fungus from terrestrial roots of an unidentified *Vanilla* sp. from the Pacific region.

Vanilla propagation under cultivation is clonal by cutting. This process reduces the genetic variability and the emergence of new genetic combinations produced by sexual reproduction[28]. In consequence, the *Vanilla* industry has a limited number of cultivars and hybrids[29]. Understanding the role of mycorrhizal fungi for stimulation of seed germination could be useful in *Vanilla* breeding programs, including the development of hybrids with better organoleptic qualities and resistance to diseases and pests[4,28-31]. Moreover, improving the sexual reproduction of *Vanilla* spp. contributes to *in situ* conservation programs and enhances the genetic variability of the endangered and underutilized phytogenetic resources[32].

Studies on seed germination and physiology require accurate information on seed viability. Traditionally, the most frequently used method to evaluate seed viability is the tetrazolium chloride method[33]. In orchid seeds, this method is useful in many species with non-coloured seed coats that allow the embryo observation[34-36]. However, *Vanilla* seeds are covered by a hard and dark coat that makes it difficult to determine the embryo viability[9,33,37,38]. Therefore, few studies on *Vanilla* seed germination have been done with an assessment of the seed viability[25,28,37]. This study proposed a new method for the evaluation of *Vanilla* seed viability, useful in germination for plant breeding and ecological and physiological studies.

As *Vanilla* is a promising resource crop in many regions in the world[1,28,39], it is important to understand biological aspects of the native species. This information includes symbiotic interactions between plants and their mycorrhizal fungi and the development of sexual symbiotic propagation in order to support conservation and re-introduction programs[6,15,28,40,41]. In this research, three *Vanilla* species with promising traits for agro-forestall production were selected from the municipality of Valle del Cauca (Colombia): *V. rivasii* Molineros, González, Flanagan and Otero, *V. calyculata* Schltr and *V. odorata* C. Presl. Our research question was if mycorrhizal fungi isolated from *Vanilla* adult plants are able to induce *Vanilla* seed germination and if there are differences among the isolates. Based on previous studies on orchid seed germination[19,20,25], our hypothesis was that *Ceratobasidium* can induce better germination than other mycorrhizal fungi. To answer this question, pure cultured mycorrhizal fungi previously isolated from three adult *Vanilla* species[30] were tested for seed germination under *in vitro* conditions.

MATERIALS AND METHODS

Procedure of the study is summarized in Fig. 1, which was divided into the following phases.

Fruit collection: Ripe fruits were obtained from wild populations of *V. rivasii*, *V. calyculata* and *V. odorata* since May, 2012 to January, 2013. All collection sites are located in Valle del Cauca region, Colombia. For *V. rivasii*, fruits were collected in forests of Buenaventura city on the Pacific coast at an altitude of 26 m a.s.l. For *Vanilla odorata*, fruits were collected in Riofrio town at an altitude of 1122 m a.s.l. Finally, for *V. calyculata* fruits were collected in Cali city at an altitude of 1034 m a.s.l.

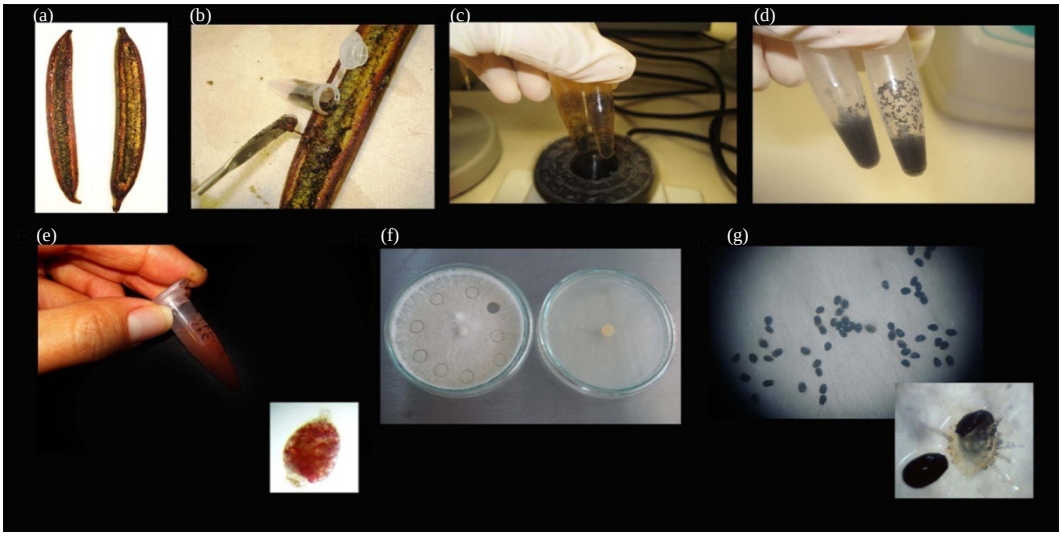

Fig. 1(a-g): Procedure of seed germination tests, (a) Collection and dissection of *Vanilla* fruits, (b) Extraction of seeds, (c, d) Cleaning of seeds, (e) Seed viability test (the box highlights a viable embryo), (f) Pre-cultured fungi isolated from the roots of the adult plant and (g) Seeds in contact with fungi: Symbiotic germination test (the box highlights an intact seed and a developed protocorm from *V. rivasii*)

Seed viability procedure: *Vanilla* fruits contain a fat sticky substance around the seeds and it is necessary to be remove it for germination experiments in order to minimize contamination. The ripe fruits were transported to the Plant Propagation Laboratory from 'Universidad del Valle' (Cali, Col.) and cleaned in a soapy solution and rinsed twice with tap water. Then, the seeds were cleaned in a 3% sodium chloride for 15 min. Under the laminar flow hood with sterile conditions, the fruits were dissected longitudinally with a sterile scalpel to extract the seeds from the medial part of the fruits, which were stored in eppendorf tubes. Then, the seeds were washed following a modified Knudson method[37]. The modification included three washing cycles as below: 95% ethanol, centrifugation 4000 rpm for 5 min and discharging the supernatant. Approximately 3% sodium-chloride solution with neutral soap was added and the seeds were shaken using a vortex mixer for 5 min and centrifuged to 4000 rpm for 5 min. After discarding the supernatant, the seeds were rinsed with sterile distilled water for three times and stored into 1.2 mL eppendorf tube with sterile water. Then, a proportion of clean seeds was placed over a sheet water resistant sandpaper number 320 and covered with another portion of the sandpaper for scratch softly the seeds for a minute by rubbing the two sandpaper sheets. After that, free embryos were observed as white dots and were transferred it to 2.0 mL eppendorf tubes with 1.0 mL of 2, 3, 5-triphenyltetrazolium chloride (TTC) 1% (Sigma Aldrich). Finally, the tubes were covered with aluminum foil for a period of 96 h. For seed viability measurement, the embryos were observed under a dissection microscopy. In positive reactions, the embryos were stained red to pink, while non-viable embryos remained unstained and translucent. Equation 1[33] was applied to calculate the percentage of viability for 1 mL of seed solution.

$$\text{Viability } (\%) = \frac{N_v}{N_t} \times 100 \quad (1)$$

where, N_v is the number of viable seeds and N_t is the number of total seeds.

This procedure was repeated for 10 times for each species and averaged the data to calculate the percentage of viability.

Seed symbiotic germination: Isolated fungi previously obtained from several *Vanilla* species[30] were transferred to petri dishes with Oat Meal Agar (OMA)[43] modified as follow: 1.0 g peptone, 4.0 g of oat powder and 10.0 g agar per 1000 mL of media (Table 1). When the fungi reached three quarters of the plate, 1.0 mL of surface-sterilized seeds suspension was added to the petri dishes[25]. The OMA without any fungi was used as negative control. An additional trail was included to test the role of glucose on the asymbiotic seed germination (positive control), seeds in OMA with 1.0 g of glucose per liter without fungi. The trails were performed using 45.0 mL glass petri dishes incubated on dark conditions at 32°C for 90 days. The percentage of

Table 1: Treatments in germination trials including type of media, each of ten strains previously isolated and two control treatments

Treatments	Growing media and fungal strain	Code strain	Isolated from
1	OMA+ *Tulasnella*	ATME024	*V. rivasii*
2	OMA+ *Tulasnella*	ATME016	*V. rivasii*
3	OMA+ *Tulasnella*	ATME102	*V. odorata*
4	OMA+ *Tulasnella*	ATME112	*V. calyculata*
5	OMA+ *Ceratobasidium*	ATME117	*V. calyculata*
6	OMA+ *Tulasnella*	ATME043	*V. odorata*
7	OMA+ *Tulasnella*	ATME046	*V. odorata*
8	OMA+ *Tulasnella*	ATME023	*V. rivasii*
9	OMA+ *Ceratobasidium*	ATME103	*V. odorata*
10	OMA+ *Ceratobasidium*	ATME015	*V. rivasii*
11	OMA without fungi (negative control)	-	-
12	OMA+glucose without fungi (simple asymbiotic media)	-	-

seed germination (% G) was calculated following Arditti method[44] modified by Pierik *et al.*[45] as given in Eq. 2:

$$G\ (\%) = \frac{N_1 + N_2 + N_3 + N_4 + N_5 + N_6}{N_0 + N_1 + N_2 + N_3 + N_4 + N_5 + N_6} \times 100 \quad (2)$$

where, N_x is the frequency of each growing stage of the embryo development. Thus, N_0 is the frequency of stage 0 (without germination), N_1 is the frequency of stage 1, N_2 is the frequency of stage 2 and so on.

From this data, the Index of Growth (IG), which measures the average stage of development[19,28] was calculated following the Eq. 3 modified from Spoerl[46]:

$$IG = \frac{N_1 + (N_2 \times 2) + (N_3 \times 3) + (N_4 \times 4) + (N_5 \times 5) + (N_6 \times 6)}{N_0 + N_1 + N_2 + N_3 + N_4 + N_5 + N_6} \quad (3)$$

where, N_0, is the number of seeds on stage 0, N_1 is the number of seeds on stage 1 and so on. In N_6, seedling reach four foliar sprouts. Each of 12 treatments (Table 1) had three replicates.

Statistical analysis: For percentage of viability variable, a one-way analysis of variance (ANOVA) were performed to test differences among species for significance ($p \leq \alpha = 0.05$) and finally a post-ANOVA Tukey test was applied. Differences among treatments for percentage of seed germination (% G) and Index of Growth (IG) variables were tested for significance ($p \leq \alpha = 0.05$), with a Generalized Linear Model (GLM) with a "Gamma" distribution and link function "Log" due to deviations from assumptions of normality and equal standard variation in spite of data transformations. All analysis were performed using the R Software, version 3.3.1[42].

RESULTS

Seed viability: For the first time, it was possible to determine the *Vanilla* seeds viability. The three *Vanilla* species had acceptable percentages of viability and presented significant differences between them ($F_{(2, 27)} = 75.11$, $p<0.005$, Fig. 2), being *Vanilla rivasii* the species with the highest value.

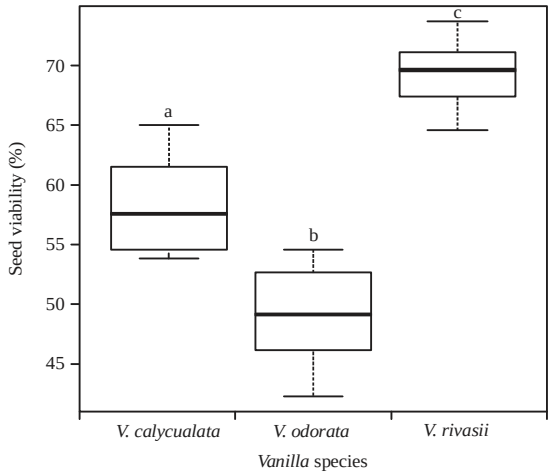

Fig. 2: Percentage of seed viability of three studied *Vanilla* spp.

Different letters correspond to treatments with significant differences (Tukey test: $F_{(2,27)} = 75.11$; $p<0.005$) (n = 10). Box plots illustrate the median (horizontal line inside each box), 25-75 percentiles (the box). The whiskers (the two lines outward from a box at each end) represent the smallest (the bottom whisker) and the largest (the top whisker) values in the data set

Symbiotic seed germination: The inoculated seeds from *V. rivasii* and *V. calyculata* germinated within 30 days after sowing. *V. odorata* seeds did not germinate with any treatment. The percentage of germination varied significantly among treatments ($p<0.005$) and among species ($p<0.005$) according to GLM. *Vanilla rivasii* seeds germinated with all treatments (except with the control treatment). However, the *Ceratobasidium* strains were more efficient at promoting germination (up to 80%) than the *Tulasnella* strains (up to 60%) (Fig. 3a). On the other hand, *V. calyculata* seeds germinated only with one *Ceratobasidium* strain (ATME103 = treatment 9) (Fig. 3b).

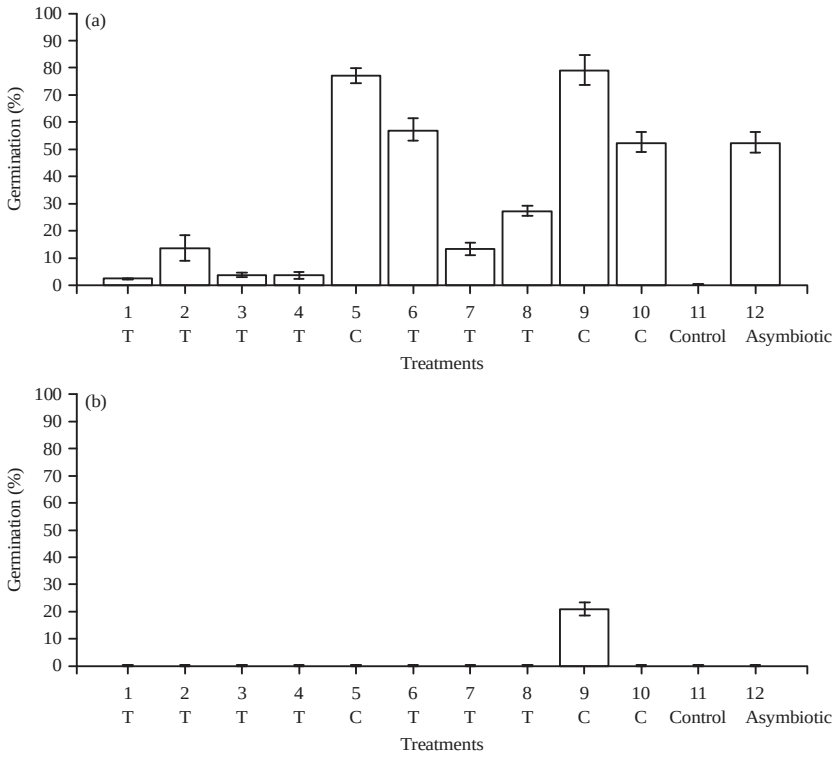

Fig. 3(a-b): Percentage of seed germination from (a) *Vanilla rivasii* and (b) *Vanilla calyculata*
The bars show the Mean±Standard Deviation ($\bar{X} \pm$ SD), C: *Ceratobasidium* strain, T: *Tulasnella* strain

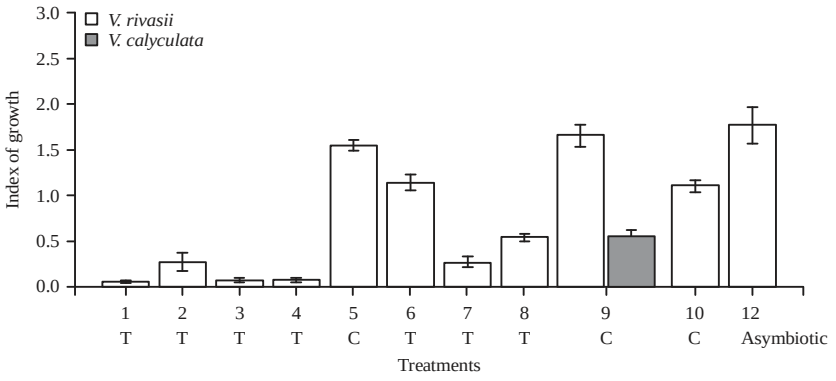

Fig. 4: Index of Growth (IG) of seeds from *V. rivasii* and *V. calyculata*
The bars show the Mean±Standard Deviation ($\bar{X} \pm$ SD), C: *Ceratobasidium* strain, T: *Tulasnella* strain

Regarding seed development stages, both *V. rivasii* and *V. calyculata* seeds reached the five stage; however, most of the seeds were in earlier development stages. Index of Growth (IG) (Fig. 4) was higher in treatments with asymbiotic medium or *Ceratobasidium* strain ATM103 treatments (treatment 12 and 9, respectively). The best germination with *Tulasnella* isolates was with strain ATM043 (treatment 6).

The stages of seed germination for *V. rivasii* (Fig. 5) and *V. calyculata* (Fig. 6) had differences in their development. Both species had five stages with differences in size of the protocorm and number and size of the rhizoids. Both species had differentiated protocorms, but *V. rivasii* developed the rhizoids since the beginning of the development (stage 1, Fig. 6) while *V. calyculata* started the rhizoid development later (stage 2, Fig. 5). These rhizoids were also thinner, longer and more abundant than in *V. rivasii*. At stage 5, *V. calyculata* had two leaves buds while *V. rivasii* had no leaves. The protocorms observed in both species had not chlorophyll pigmentations.

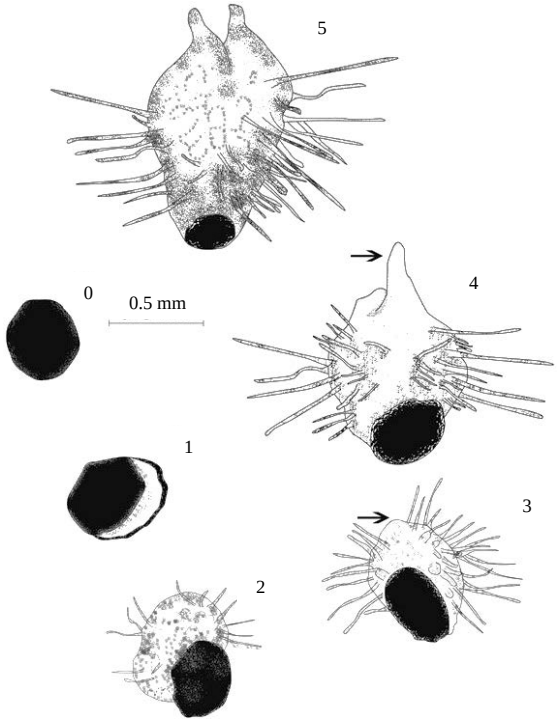

Fig. 5: Seed germination and seedling developmental growth stages of *V. calyculata*

Stage 0: No germination, 1: Rupture of the seed coat (germination), 2: Enlarging embryo, production of rhizoids and protocorm development, 3: Protocorm and rhizoids increase and appearance of the shoot (protomeristem) as denoted by the arrow, 4: Embryo has increased in size four times and enlarging meristematic zone, 5: Emergence of two leaf primordia from shoot region. Scale bar = 0.5 mm

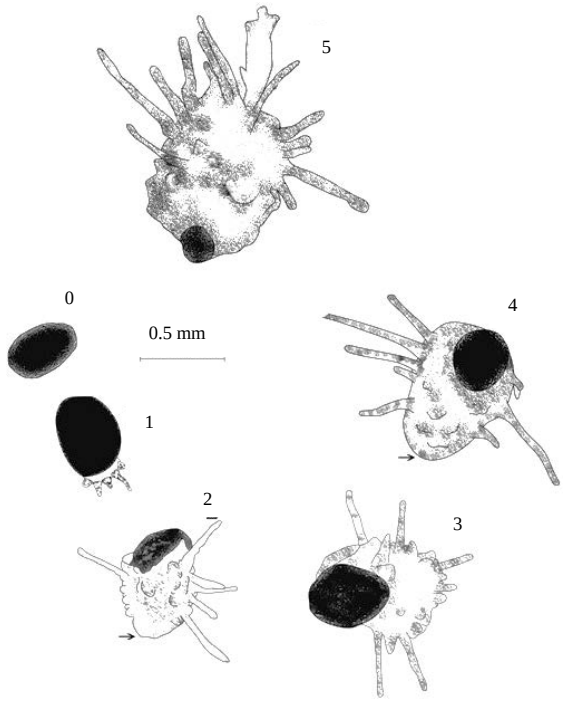

Fig. 6: Seed germination and seedling developmental growth stages of *V. rivasii*

Stage 0: No germination, 1: Rupture of the seed coat with production of rhizoids (germination), 2: Enlarging embryo and protocorm development, 3: Protocorm and rhizoids increase and appearance of the shoot (protomeristem), 4: Embryo has increased in size four times and enlarging meristematic zone as denoted by the arrow, (5) Emergence of one leaf primordium from shoot region. Scale bar = 0.5 mm

DISCUSSION

This study presented a new procedure for measuring *Vanilla* seed viability. The factors influencing orchid seed germination includes the contact of the orchid seeds with a compatible mycorrhizal fungus[15,41]. Nevertheless, non-viable seeds are not able to germinate even with compatible fungi. In consequence, evaluation of seed viability is essential to study orchid seed germination. Previous studies on *Vanilla* seed germination[25,28,37] assumed it was no possible to use TCC procedure because the seed coat of *Vanilla* seeds is too dark to allow the observation of stained embryos. Here, it is proposed a technique to remove the seed coat, which allows direct contact of the embryo with the salt.

The viability test showed that all species had acceptable viability percentages to allow the seed germination. However, *V. odorata* did not germinate with any treatment and *V. calyculata* only germinated with one treatment. This fact suggest that all species have different needs for the germination process. For example, the lack of the appropriate fungus to establish the mycorrhizal relationship and/or the lack of nutrients in the medium that allow the seed germination for these particular *Vanilla* species[47].

This study also provided relevant information about the developmental process of the protocorm of the *Vanilla* species studied. Characterizations of developmental stages in the germinative process of *Vanilla* species are scarce. However, germination experiments with asymbiotic techniques have reported more information about embryo development than symbiotic procedures. Knudson[37] realized the first and more complete characterization of seed development for *Vanilla planifolia* species under asymbiotic seed germination. In that study, the author presented six stages of the embryo development, as well as, three stages of seedling development. Menchaca *et al*.[28] studied seed germination in *Vanilla planifolia* and *V. pompona* Schiede hybrids and reported two stages of seed germination: rupture of the seed coat and complete embryo emergency however, no morphological description was performed in this study. A previous study to the present study showed the symbiotic germination process in *Vanilla* spp[25]. That study only reported the rupture of the seed coat as an indication of germination without reaching advanced stages of development. In contrast, the present study report five stages of seed development for *V. calyculata* and *V. rivasii* (Fig. 5, 6). The differences in seed development may suggest different physiological needs in relation to photosynthetic processes of adult plants[48]. For example, the development of two leaf primordia in *V. calyculata* can be indicative of an independence of the fungus when embryo beginning a photosynthetic activity from an early period. In contrast, the development of a non-photosynthetic stem in *V. rivasii* may indicate a dependence on the nutrients provided by the mycorrhizal fungus until a more advanced stage of leaf development.

It was found that *Ceratobasidium* isolates were more efficient inducing *Vanilla* seed germination than *Tulasnella* strains. These results support previous observations for other *Vanilla* species[25] and other orchids such as *Ionopsis utricularioides* (Sw.) Lindl.[21] and *Comparettia falcata* Poepp. and Endl.[49]. Nevertheless, the isolates of *Tulasnella* were able to induced *Vanilla* seed germination as well. That result contrast with previous studies in *Vanilla* species[25], but it is consistent with studies of other orchid species in which *Tulasnella* fungus induce an efficient seed germination[50-52].

Orchid's seed germination in field conditions is difficult to study because orchid seeds are small[16]. For *Vanilla* species, it is not the exception; *Vanilla* seedlings are very uncommon in wild ecosystems[48]. An alternative to evaluate the mycorrhizal potential of the fungi isolated from adult plants is to test *in vitro* seed germination. Nevertheless, not necessarily the fungi that are present in adult plants can induce seed germination. Regarding to germination and seed development of the mycorrhizal interaction of studied *Vanilla* spp., *V. rivasii* germinated with *Ceratobasidium* ATM103 (isolated from *V. odorata* roots tissue) and ATM117 (isolated from *V. calyculata* roots tissue). In contrast, *V. calyculata* seeds only germinated with *Ceratobasidium* isolated from *V. odorata* (ATM103). Finally, *V. odorata* did not germinated with any tested fungi. These results support the idea that orchid seeds share their mycorrizal fungi instead of have specificity in their relationships[18,42,25,53,54].

In germination experiments, although it was possible to observe seeds with advanced developmental stages, most seeds had a limited development with the stage 2 as the more common phase. This phenomenon has been reported for other orchid species in symbiotic germination trials[50]. Interesting, seeds of *V. rivasii* reached its most developed stage with the asymbiotic treatment with glucose. This finding is remarkable considering that the asymbiotic medium used in this study was a simple and economical medium. The asymbiotic media developed for orchids are usually complex and with specific nutritional requirements depending on the orchid species. For *Vanilla*, Knudson's pioneering work[37] evidences the need to use calcium, magnesium, iron and potassium salts, as well as sugars. Menchaca *et al*.[28] have used commercial mediums or Murashige and Skoog formula enriched with glutamine and adenine sulfate. The elaboration

of complex media is an expensive and costly procedure, especially if a large-scale propagation is to be developed. Thus, the asymbiotic medium proposed here could act as a basic medium of asymbiotic seed propagation. On the other hand, it is worth noting that asymbiotic treatments had more chance to have fungal or bacterial contamination than those with symbiotic fungi. This phenomenon has been reported previously for *Vanilla*[25]and other orchids[12,55]. Furthermore, symbiotic techniques have the advantage that mycorrhizal plants are often stronger and resistant to infections than plants asymbiotically grown. For example, Otero and Bayman[20] working with the orchid *Tolumnia variegata* (Sw.) Braem, reported that symbiotic germination methods produced more developed embryos than asymbiotic germination methods.

Within the limitations of the study, it can be mentioned the difficulty to culture all the fungi from adult plant since some orchid mycorrhizal fungi do not grow in culture. Therefore, it is possible that fungi necessary for the germination of the seeds simply could not be cultivated or evaluated in the germination tests. Furthermore, it is possible that fungus, which allow germination, is different from that needed for the advanced stages of development. As a recommendation, it is necessary to continue with germination experiments that allow reaching to phase of transplanting symbiotic seedlings to understand the whole development of the different *Vanilla* species. In addition, promoting a bank of orchid mycorrhizal fungi would facilitate research and the consequent application for commercial purposes.

Symbiotic seed germination is a significant part of orchid conservation programs[15,28,42,56]. However, *in vitro* cultivation of orchids from seed is difficult since it requires laboratory conditions. Research in independent methods from the laboratory that simulate the natural conditions of the germination represent an interesting challenge.

This study proposed an easy technique for measuring *Vanilla* seed viability given that previous germination experiments with *Vanilla* seeds[25] have been made without knowing the percentages of viability. Similarly, this study is the first in report successfully the symbiotic germination of *Vanilla* seeds with advanced stages of development after the rupture of the seed coat and the first characterization of these stages. The proposed method to test seed viability, as well as, germination process results, may have future applications on programs based on sexual reproduction, such as plant breeding of *Vanilla* species, opening the opportunity to develop more easily new hybrids for the vanilla industry. Similarly, this research provides important elements for conservation programs.

CONCLUSION

Seed development of *Vanilla calyculata* and *V. rivasii* was characterized into five stages that presented differences between the species. Although, germination was achieved for both *Ceratobasidium* and *Tulasnella* isolates, *Ceratobasidium* strains were more efficient at promoting *Vanilla* seed germination. At the level of *Vanilla* species, *V. rivasii* was the species with highest seed viability and highest percentage of germination. Results of this study may have future applications for *Vanilla* plant breeding programs, opening the opportunity to develop new hybrids for the vanilla industry, as well as, for germplasm conservation of this important resource.

SIGNIFICANCE STATEMENTS

This study presented a new procedure for measuring seed viability of *Vanilla*. The proposed technique could be a very useful method in conservation programs based on sexual reproduction, as well as, in plant breeding programs of *Vanilla* species.

It is the first successful report of *Vanilla* seed symbiotic germination with advanced stages of development of protocorm and the first characterization of these stages. This information may have future applications for *Vanilla* plant breeding opening the opportunity to develop more easily new hybrids for the vanilla industry, as well as, for germplasm conservation. As a recommendation, it is necessary to continue with germination experiments that allow reaching to phase of transplanting symbiotic seedlings to understand the whole development of the different *Vanilla* species.

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