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Research Article

Preliminary Evaluation of the Potential of Macromycetes as Mediators in Bio-Hardening of Orchid Plantlets

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Abstract

The most efficient technique for the massive multiplication of orchids is micropropagation *in vitro*. Once plants are extracted from their containers they encounter abrupt changes in their environmental conditions, resulting in low survival rates. To avoid high levels of plant mortality an adaptation period called hardening is required. Conventional approaches for the hardening process apply expensive chemical substances such as pesticides and fertilizers that generate toxic residues. Alternative methodologies have explored the use of microorganisms to assist the adaptation phase, this is called bio-hardening. However, there are no reported scientific studies on the use of macro fungi in bio-hardening to the knowledge of the authors.

The aim of this work is to explore the potential of macro fungi as bio-hardening agents of orchids. For this purpose, 5 macromycetes fungi where evaluated by co- culture to assist in the bio-hardening process of asymbiotically raised seedlings of orchids. Two orchid hybrids (Miltoniopsis Kumbia and LC. Irene Finney) and one species (*Catasetum tabulare*) where evaluated under co-culture treatments and a sterile substrate control during hardening. The results proved an enhanced development and improved survival rates for the co-cultured plantlets in comparison to the control. Survival rates are similar or better in comparison to other conventional and bio-hardening techniques mentioned in scientific studies. This is the first report to the knowledge of the authors of macromycetes used as bio-hardening agents of vitro plants and poses a new sustainable clean alternative for the ornamental plant industry.

Keywords: Bio Hardening; Orchids; Macro Fungi; *In vitro*; *Ex vitro*; PGR; Cattleya; Miltoniopsis; Catasetum

Introduction

In vitro propagation is the most efficient technique for massive multiplication of plants, including orchids. During *in vitro* development, conditions are controlled and constant. When the plants are extracted from their containers, they phase abrupt changes in their environmental conditions resulting in a low survival rate. To avoid the high mortality of plants during this stage of the productive process it is required that said seedlings go through a period of adaptation called hardening. During this process the seedlings condition their physiology to the new environment (variations of temperature, luminosity, relative humidity and the presence of predatory and pathogenic organisms), the lack of this variations during the *in vitro* stage generate distortions in the development of the plants and repression or modulation of some metabolic routes. In accordance with the above, vitro plants have a reduced photosynthetic capacity, malfunction of the stomata, an altered root system (with fewer or without root hairs), a considerable decrease in osmotic potential and a low wax reserve with poor development of the cuticle [1].

The conventional approach to the hardening process requires the use of agrochemicals like fungicides, insecticides and bactericides, which complement the control of humidity, temperature and luminosity [2]. Although the use of agrochemicals during the adaptation stage can prevent the infection of seedlings by pathogens and pests, it does not solve the problem of susceptibility that plants face when lacking a beneficial fauna in their rhizosphere [3], nor it activates the synthesis and expression of plant defense mechanisms by means of metabolites such as phytoalexins, is flavonoids, amongst others, that provide protection against biotic and abiotic stress [4]. The use of agrochemicals also generates important costs in the processes of mass production of plants [2] and is also related to cause health and environmental problems due to contaminant residues [5-7].

In order to overcome the difficulties concerning the use of these substances during hardening, alternative methodologies have been explored to assist plants during their adaptation, exploiting the beneficial relationships between microorganisms and plants, thus originating a new technique called bio-hardening [7-10]. In general, studies related to this technique have focused on the use of bacteria [2] and micro fungi [8,9,11], with good results [12]. Although the use of micromycetes in bio-hardening has allowed the improvement in the survival rates, vigor of the plants and other attributes [11], these organisms present some disadvantages as are; the low commercial access to strains, the difficulty of taxonomic identification by non-molecular methods [13], the specificity of habitat and host interaction [3], a high production of mycotoxins

[14] and the high number of this type of fungi reported as plant pathogens [9,14].

Although to date there are no reports of the use of macro fungi in bio-hardening processes, it is widely known that these organisms produce many secondary metabolites with recognized biological actions such as plant growth regulators, triterpenoids, polysaccharides and in general beneficial compounds for the development of plants (Table 1), therefore it may be inferred that they can present a great potential for use as mediators in bio-hardening.

Macromycete	Metabolite	Biological Activity	Reference
Ganoderma sp.	Ganoderic triterpenes. Triterpenoids of Ergosta and Lanosta type.	Immunomodulator Antimicrobial	[15]
Boletus impolitus	Gibberellins	PGR	[16]
Suillus granulatus	Gibberellins	PGR	[16]
Fomitopsis sp.	Triterpenoids of Lanosta type.	Antimicrobial	[17]
Coprinus atramentarius	Gibberellins	PGR	[18]
Laetiporus sulphureus	Triterpenoids of Lanosta type.	Antimicrobial Antiviral Immunomodulator	[19]
Cortinarius Fr. sp.	Gibberellins	PGR	[18]
Lentinus tigrinus (Bull.: Fr.)	Gibberellins and Abscisic acid (ABA)	PGR	[20]
Inonotus obliquus	Triterpenoids of Lanosta type.	Antitumor Antimicrobial Antioxidant	[19]
Gibberella fujikuroi	Gibberellins	PGR	[16]
Phellinus pini	Vinylphenols	Antimicrobial	[17]
Pleurotus florida	Abscisic acid (ABA)	PGR	[21]
Pleurotus Ostreatus	Gibberellins	PGR	[16]
Lentinula edodes	Polysaccharides	Immunomodulator	[22]

Table 1: Secondary metabolites isolated from macromycetes with biological activities potentially useful in bio-hardening.

Based on the above, the present investigation intends to evaluate through co-cultures, the potential that macromycetes have as mediators in bio-hardening. For this purpose, 5 edible, saprophytes, cellulolytic and ligninolytic fungi were selected [23], a condition that provides them of an important advantage in the case of hardening orchid seedlings, since the latter are grown on pine bark, a substrate consisting mainly of cellulose and lignin. On the other hand, none of the fungi evaluated have been reported as a phytopathogens, on the contrary, the metabolites extracted from these fungi have shown to have anti-fungal, bacteriostatic and bactericidal biological activity [24].

Materials and Methods

Fungal inoculate

The fungal species used in this study were *Pleurotus ostreatus* Var. Columbinus, *Pleurotus citrinopileatus, Lentínula edodes, Laetiporus sulphureus* and *Agaricus bisporus*. The strains for Portobello, Shitake and Oyster mushrooms were purchased from Shroom Supply, while the *Laetiporus sulphureus* strain was isolated from a fragment of a specimen collected in the eastern hills of Bogotá, Colombia, growing on a stump of eucalyptus tree.

Plant material

Seedlings from 2 hybrids Cattleya (LC. Irene Finney), Miltoniopsis (Miltoniopsis Kumbia) and one specie Catasetum (*Catasetum tubulare*), belonging to 3 relatively distant genus, all native to Colombia where produced by asimbiotich seed germination *in vitro.*

A symbiotic seed germination under *in vitro* conditions was done using the enriched culture medium standardized by Murashige and Skoog (MS medium) modified with 100 ml of coconut water per liter of medium [25]. These seeds were produced by directed crossings of plants belonging to a private orchids collection, as part of the plant breeding program. The flowers of each of the plants were fertilized by cross-pollination, extracting the pollen masses (pollinia) of one flower and depositing them in the receptive stigma of another. The capsules where harvested just before reaching dehiscence ("Green pod" or green capsule method). Harvested capsules where washed with running tap water and soap, later they were superficially disinfected with a calcium chloride solution at a concentration of 0.7 mg/ml [26], leaving them to rest for 7 minutes in the sterilizing solution, under aseptic conditions [26,27]. Afterwards capsules where dissected seeds extracted and planted in sterilized jars served with the culture medium under the laminar air flow cabinet.

Orchid seeds require different periods for germination under *in vitro* conditions according to the species. A general range between one to six months can provide a satisfactory number of germinated seeds for the three evaluated lines.

The seedlings were kept *in vitro* in 50 ml of modified MS culture medium, replanting them every 4 months. These orchids require at least 3 movements to reach the ideal size for hardening. After 1 year of cultivation *in vitro* from the sowing of the seed, the seedlings developed in to an ideal size with a sufficiently developed root system, necessary to resist the hardening period.

Co-culture

For the co-cultures bio-assays, the seedlings were individualized, any residue of enriched medium present in the roots was washed off and any undifferentiated fragments of callus or protocorm was discarded. Individual plants were sorted by size.

Fragments of *Pinus patula* bark of approximate size 0.5 cm x 0.5 cm were used as substrate. Substrate was soaked during 12

hours in distilled water. The excess water was discarded and the substrate was vapor sterilized for 1 hour in the autoclave. Once the substrate was at room temperature, it was inoculated with the spawn of the different fungi evaluated. When each substrate was completely colonized by mycelium (mycogenized substrate), reusable plastic trays of 72 cells were filled with the colonized substrate to house the seedlings for the bio-hardening trial. The mycogenized substrate was added to each cell, 1 seedling per alveolus was placed according to the quantities indicated on table 2 during a period of 4 months.

Treatment	Number of Miltoniopsis Kumbia plantlets	Number of Catasetum tubulare plantlets	Number of LC. Irene Finney Plantlets
Control*	20	10	10
P. columbinus	50	50	20
P. citrinopileatus	10	10	15
L. edodes	20	10	20
L. sulphureus	50	50	20
A. bisporus	15	15	15
Total	165	145	100

Table 2: Number of plantlets sowed per treatment.
* Sterile substrate.

The planted trays were placed in the greenhouse, under an irrigation regime of 15 minutes of micro-sprinkling every 6 hours with tap water, without any additional treatment. The relative humidity was controlled keep it as near as possible to 80%. The seedlings were left for 4 months to compare the development and survival under each treatment. No chemical agent was used during the hardening process to control pests and diseases, nor was any fertilizer applied during the evaluation of the treatments. The control of humidity by means of nebulized irrigation, the reduction of radiation with artificial shade and the physical barrier of the greenhouse were the only abiotic measures used during this study.

Results

After a 4-month period of the hardening process, the individuals who survived were counted (Table 3), thus obtaining the survival rate for each treatment (Table 4).

Treatment	# of hardened plantlets of Miltoniopsis Kumbia	# of hardened plantlets of Catasetum tubulare	# of hardened plantlets of LC. Irene Finney
Control	0	5	4
P. columbinus	46	50	20
P. citrinopileatus	10	10	14
L. edodes	18	10	20
L. sulphureus	47	42	18
A. bisporus	16	13	13

Table 3: Number of surviving individuals after the hardening process in each treatment.

Treatment	Miltoniopsis Kumbia (%)	Catasetum tubulare (%)	LC Irene Finney (%)
Control	0	50	40
P. ostreatus var. Columbinus	92	100	100
P. citrinopileatus	100	100	93
L. edodes	90	100	100
L. sulphureus	94	84	90
A. bisporus	100	87	87

Table 4: Survival rate of each orchid line per treatment.

Due to the high relative humidity maintained in the greenhouse, a spontaneous colonization of bryophytes and ferns occurred. The presence of healthy moss is evidence of the non-application of agrochemicals and fertilizers. This accompanying flora was not eradicated since they do not affect negatively the development of the orchids and generate a desirable microclimate for their growth.

The survival rate was not the only factor that varied between the treatments and the control, the vigor of the plants that developed on the substrate colonized by the fungi evaluated was higher, evidenced by a faster growth in comparison with the control (Figure 1). Likewise, the seedlings of the control treatment suffered from symptoms of desiccation, slow development and had a high incidence of pests and diseases (Figure 2). Since no agrochemicals or fertilizers were used during the adaptation process, the positive results, both in the survival and in the development of the treated plants, are a real discovery and show a great potential for this method of bio-hardening.

Figure 1: LC Irene Finney plantlets after 2 years of development. A) Plantlet hardened under co- culture with *P. ostreatus* Var. Columbinus. B) LC Plantlet after 2 years of development with no co-culture treatment.

Figure 2: Control hardened plantlets showing symptoms. A) LC Irene Finney plantlet with slow growth. B) Miltoniopsis Kumbia plantlet with mollusk predation signs. C) *Catasetum tabulare* dehydrated plantlet.

Figure 3: LC. Irene Finney in co-culture with *P. ostreatus* Var. Columbinus.

Figure 4: LC. Irene Finney plantlets after 2 years of trial in the Green house. A) Control with no co-culture. B) Co-cultured plantlets.

Discussion

The colonization of the substrate by Pleurotus genus was considerably faster than that of the others (unpublished data). Likewise, the species *P. ostreatus* has the additional advantage in comparison to *P. citrinopileatus* that it develops better in the climatic conditions of Bogotá (average 14°C), which facilitates its production in the laboratory, also presenting a greater potential for its use as a bio-hardener under the conditions of the place of study. Regarding the process itself, the results obtained show in all cases that the seedlings growing on the mycelium colonized substrate demonstrated a better adaptation to the *ex vitro* conditions, as reflected in the survival rate of each treatment that can be compared to reported survival rates of other published work (Table 5), showing that the macromycetes exhibit a good potential to mediate bio-hardening processes and that their action seems to be general for all the evaluated orchids.

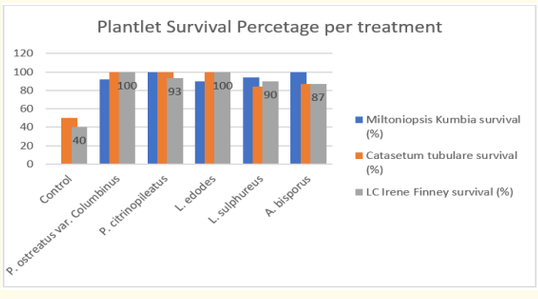

Figure 5: Plantlet survival percentage per treatment.

The differences amongst the survival rates between each co-culture treatment where very small, so no real affirmation about which macro fungi is better for bio hardening of these orchids can be concluded based on the survival rates. Also, more replicas are required to be able to make a serious statistical analysis of this results.

On the other hand, the differences between the survival rates of each treatment and the control are very marked. Even with no statistical analysis it is easy to see that in every case the survival rate was considerably higher when orchids where hardened through co-culture with the macro fungi.

If compared with other reported survival rates (Table 5), it can be inferred that this bio-hardening methodology shows promising results, furthermore considering that most of the reported protocols use either toxic substances (agro-chemicals), unsustainable substrate (moss) or expensive treatments (MS salts, hardening chambers) that could difficult the scaling up of the process form commercial means.

In the present time it is widely recognized that mycorrhiza provides the plant with an enhanced access to nutrients, disease resistance and a greater tolerance to abiotic stress (dehydration) as well as tolerance of some biotic stress [1], for this reason the use of this class of mycorrhizal forming fungi has been a subject of intensive study [35]. Apart from *L. edodes*, a mushroom whose mycorrhizal association was determined with the orchid *Erythrorchis ochobiensis* (Umata, H., 1997), the evaluated fungi have not been reported as mycorrhiza forming fungi with orchids, even more so to those belonging to the genus used in this trial. This allows us to infer that this association is not required to obtain a good effect when macromycetes are used as mediators in bio-hardening processes and that, even without ruling out the possibility of mycorrhization, the benefits that seedlings gain from this type of fungi are due to the production by the fungi of secondary metabolites with recognized biological activities such as: sterols and fatty acids that exhibit antimicrobial, antifungal and immunostimulatory activity, diterpenes such as gibberellins with phytohormonal activity and polysaccharides that provide a source of energy and act as immunostimulant. From the above the first ones would be responsible for the resistance of the seedlings to the attack of pathogenic microorganisms, while the second metabolites mentioned would have effects on the development of the seedlings, since in a first stage they would increase the mitotic rate of caulinar and radicular tissue. In the same way, the formation of hyphae networks on the substrate increase the capacity of water retention in the substrate, thus decreasing the loss of plants by dehydration.

Subsequent studies should focus on structurally determining the fungal metabolites biosynthesized by the macromycetes studied in order to establish their real contribution to the bio-hardening process. Likewise, corroborate or rule out the possible formation of mycorrhizal associations between these fungi and orchids, through the inoculation of symbiont lacking orchids with the

Orchid species	Type of hardening	Treatment	Survival rate %	Reference
Cattleya trianae	Conventional	Preventive use of fungicide and MS salt applications during the first month.	80 – 85	Franco., *et al*. [28]
Phalaenopsis y *Cattleya*	Conventional	Pre-hardening *In vitro* and MS salts aplication during the first month.	90 – 100	Díaz., *et al*. [29]; Deb., *et al*. [2]
Laelia speciosa	Conventional	Pre-hardening *In vitro* and acclimatization chamber.	97.5 – 100	Ortega-Loeza., *et al*. [30]
Dendrobium chrysanthum	Conventional	Pre-hardening *In vitro.*	79	Hajong., *et al*. [31]
Aerides ringens	Conventional	Captan 80 WG fungicide preventive application and hardening chamber.	80	Srivastava., *et al*. 2015
Camellia sinensis	Bio-hardening	*Trichoderma harzianum* *Trichoderma harzianum*, *Pseudomona fluorescens* and *Azospirillum brasilense*	>70 81	Thomas., *et al*. [32]
Cattleya walkeriana	Bio-hardening	Bacteria from the genus *Enterobacter* and *Bacillus*	75 y 80	Galdiano Júnior., *et al*. [33]
Vanda coerulea	Bio-hardening	*Rhizoctonia zeae*	80	Aggarwal., *et al*. [34]
Cymbid Ium	Bio-hardening	Endophytic fungi isolated from the species *Aerides multiflorum* and *Rhynchostylis retusa*	92 – 95	Hossain., *et al*. [4]

Table 5: Orchid survival rates reported in cited literature, both conventional and bio-hardening methodologies.

macromycetes evaluated, confirmation of peloton formation in the roots, isolation of the fungi from the pelotons and confirmation of the identity of the fungi by means of their microscopic characterization and fruiting, thus fulfilling one of Koch´s postulates [35].

Conclusions

Although the use of microorganisms has been explored in bio-hardening processes, this is the first report to the knowledge of the authors of the use of macromycetes fungi for this purpose. The effects evidenced are: higher or equal survival rates to those reported for both conventional and bio-hardening techniques, greater vigor of the plants under co-culture treatment in comparison to control treatment, total absence of symptoms of desiccation, pests and diseases in the treated plantlets, coupled with the absence of agrochemical and fertilizer applications, indicating that the macromycetes evaluated have great potential as hardening mediating agents for orchid seedlings, which could lead to the development of a novel "clean" technology of bio-hardening, since it does not use xenobiotic ingredients (agrochemicals), based on a simple, easily scalable and sustainable pioneering methodology. Linked to the above is the fact that said effects on orchids do not seem to be species-dependent, unlike mycorrhizal relations, which is an advantage of this methodology over pre-existing bio-hardening techniques.

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