



Evaluation of protocorm formation in meristematic regions of roots and leaves of *Miltonia spectabilis moreliana* (Orchidaceae).

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ABSTRACT- The aim of this study was to evaluate the effect of 2.4-D and BAP on protocorm formation in meristematic regions of root and leaf of *Miltonia spectabilis moreliana*, and to test the effect of sucrose concentrations and pH levels on the growth of protocorms. Root and leaf segments of *in vitro*-germinated plants were cultured in MS/2 culture medium supplemented with 2.4-D and BAP, and kept in the dark for 210 days. The combination of 3 mg.L⁻¹ 2.4-D and 1 or 3 mg.L⁻¹ BAP provided the best results. The concentrations of 15 and 30 g.L⁻¹ sucrose, independently of the pH, were more efficient for the *in vitro* culture of this species, as they promoted an increase in the number and length of roots, height, and fresh weight of the plantlets. The pH was significant only in combination with 15 g.L⁻¹ sucrose for the length of the largest root and fresh weight.

Key Words: Growth regulators, Orchidaceae, pH, Sucrose.

Introduction

Orchidaceae, one of the greatest families of angiosperms, includes about 780 genera and 25,000 species [1] particularly well represented in tropical and subtropical environments [2]. Due to their ornamental characteristics, these plants are very valued by both orchid enthusiasts and the general flower market consumer as a whole. In the neotropics, the traditional gathering of indigenous orchid species and the consequent trade market, along with the destruction of their natural habitat brought about by expansion of agriculture resulted in many species becoming endangered or extinct [3]. Among such species is the *Miltonia spectabilis* Lindl. var. *moreliana* Henfr., an epiphyte orchid native to Brazil [4].

Therefore, the use of techniques allowing the production of a large number of plants in a short period of time, i.e. in a shorter period when compared to the conventional methods of propagation, can be useful to repopulate the natural habitats. In this context, tissue culture can be a good alternative to achieve the aforementioned goal [5].

Research studies using both radicular apex and young leaves of orchids have shown that when in suitable conditions of culture medium, portions of organs can be induced to form callus and protocorms allowing a large scale production of superior plants [6, 7]. Protocorm is a term used to designate a stage of development of the embryo of orchids [8, 9].

The nutritive medium must provide all essential substances for tissue growth and *in vitro* development. Despite the fact that the saline formulation of Knudson [10] is currently the most used medium for *in vitro* propagation of orchids, several authors [7, 11, 12, 13] have obtained good results with the use of the MS culture medium [14]. However, it is necessary to determine the optimal conditions in terms of nutrients, medium pH, regulators and carbohydrates required by each variety [15]. Growth regulating substances such as the auxins and cytokinins are usually added to the culture medium to help *in vitro* development, thus influencing cell division and stretching [5].

Among the components of culture medium are also carbohydrates, which provide metabolic energy and carbon skeletons for amino acid and protein biosynthesis, structural polysaccharides and organic compounds required for cell growth to occur. Sucrose is the most used carbohydrate, and its concentration is an important factor for optimal explants growth [16]. Orchids usually grow better in a slightly acid medium [17], consequently the effect of a medium pH has interested researchers due to its direct role in the availability of nutrients contained in media [18].

Studies aiming to obtain a regenerative protocol for *M. spectabilis* var. *moreliana* are scarce. There are reports of *Miltonia* sp regeneration from pseudobulbs [19], and through the use of meristem culture medium [20] cited by [8]. The aim of this study was to evaluate the effect of different concentrations of 2.4-D and BAP



on protocorm formation in meristematic regions of root and leaf segments of *Miltonia spectabilis* var. *moreliana*, and to test the effect of different sucrose concentrations and pH levels on *in vitro* protocorm development.

MATERIAL AND METHODS

Vegetal material and culture conditions

The experiments were conducted at the Botanical and Environmental Analysis Laboratory of Centro Universitário Hermínio Ometto, Araras, SP, Brazil.

Seeds of *M. spectabilis* var. *moreliana* taken from immature orchid capsules, after being disinfected, were inoculated in glass flasks (200 mL) containing 40 mL of half strength MS basal culture medium (MS/2) supplemented with sucrose (30 g.L⁻¹), MS vitamins and agar (6.5 g.L⁻¹). The cultures were kept in a growth room with a temperature of 25 ± 2°C, with a 16-hour photoperiod, under irradiance of 40 μmol.m⁻².s⁻¹ supplied by white fluorescent light bulbs. In order to assess the regulatory growth effects on the formation of protocorms, the cultures were inoculated in test tubes (22 x 150 mm) containing 10 mL of medium and incubated in the dark. For assessing the effect of sucrose and pH on protocorm growth, the cultures were transferred to flasks containing 40 mL of medium under light [3].

Regulatory effects of the growth on protocorm formation

Foliar and radicular apices (0.5 cm) originating in these plants germinated *in vitro* and with approximately 18 months of cultivation were inoculated in MS/2 basal medium supplemented with different combinations of 2,4-D-acid dichlorophenoxyacetic (0.3 and 30 mg.L⁻¹) and BAP - benzilaminopurin (0.1 and 3 mg.L⁻¹). The experimental delineation was totally randomized with ten repetitions. Each experimental unit was comprised of a test tube with three explants [3].

Observation of morphogenetic modification was carried out every 15 days during the 210 day-period of cultivation. After that period, the material was exposed to irradiance of 40 μmol.m⁻².s⁻¹ for a 16-hour photoperiod, remaining in this condition for 30 days. The explants responding to the process were transferred to new test tubes containing growth regulator-free basal medium where they were kept for 120 more days, being replated every 30 days. After this period, the percentage of radicular and foliar explants developed into protocorms and the number of protocorms formed by each explant were recorded [3].

Effect of sucrose and pH on *in vitro* growth of protocorms

Protocorms of about 0.3 cm and 0.001 g, grown from foliar segments and radicular apices cultured in basal medium supplemented with 2,4-D (3 mg.L⁻¹) and BAP (1 mg.L⁻¹), were used as explants. These were transferred into flasks containing basal medium supplemented with different concentrations of sucrose (0, 15, 30, 45 and 60 g L⁻¹) and pH (5.0, 5.8 and 6.6) adjusted with the use of sodium hydroxide (NaOH) or chloridric acid (HCl) at 1N, before the addition of agar [3].

The experimental delineation used was randomized with five repetitions. The experimental unit consisted of a flask with ten explants. The plantlets were cultured for 180 days, and replated on the 90th day. An evaluation of the following variables was carried out, i.e. height of aerial portion; number of roots, length of the longest root, number of leaves, and weight of fresh matter. The data obtained were submitted to an analysis of variance analysis using the statistic software SISVAR (Variance Analysis System for Balanced Data) [21] and Scott-Knott comparison of averages test at 5% significance.

RESULTS AND DISCUSSION

All results obtained in this paper were very similar at reported by [3] to *Miltonia flavescens* Lindl., suggesting a possible physiological uniformity for the genera.

Effect of growth regulators on the formation of protocorms

All the explants remained green while cultured in the dark in MS/2 basal medium with or without 2,4-D and BAP. During the first 45 days of culture, there wasn't any callus formation on the explants in any of the cultures. There was, however, a slight tumefaction on the radicular apices, as reported by [3] to *Miltonia flavescens* Lindl.

The first modifications in the radicular apices cultured *in vitro* were first observed around the 60th day after culturing. Some explants cultured in basal medium supplemented with 2,4-D and BAP presented some yellowish-white nodular mass on the very edge of the scalpel cuts after 210 days on which it was possible to notice the development of protocorms. [6] reported finding a similar response when working with *Oncidium*



varicosum. In Kerbauy's study [6] protocorms originated from apical root segments after 210 days of being cultured in medium supplemented with ANA (1 and 2 mg.L⁻¹) and BAP (0.05 mg.L⁻¹).

Visible morphological responses in foliar apices became evident 60 days after the tissue culture had commenced. It was possible to notice the formation of protocorms without the formation of nodular mass on the cut edges of the explants cultured in growth regulator-free medium, and 30 days later, a complete orchid plantlet had originated. Nodular masses also became visible on the explants 60 days after the initiation of the culture in medium supplemented with 2.4-D (3 mg.L⁻¹) and BAP (1 or 3 mg.L⁻¹) on the cut area of the explant, as reported in study with *Miltonia flavescens* Lindl. [3].

The addition of 3 mg.L⁻¹ of synthetic auxin 2.4-D had a remarkable effect on the formation of nodular masses which, afterwards, originated protocorms. Its combination with BAP (1 or 3 mg.L⁻¹) was important because it allowed greater induction of nodular masses not only from foliar segments, but also from radicular apices (Table 1). The larger number of protocorms using radicular apices (205) was obtained with the combination 3 mg.L⁻¹ of 2.4-D and 3 mg.L⁻¹ of BAP, while the combination 3 mg.L⁻¹ of 2.4-D and 1 mg.L⁻¹ of BAP allowed the induction of a larger number of protocorms from foliar segments (221). In both cases, however, the proportion of explants with protocorms was low, decreasing the average of protocorms per explants, i.e. 5.1 and 6.4, respectively.

The results obtained were similar to those reported by [10] for *Oncidium* 'Gower Ramsey' and [3] for *Miltonia flavescens* Lindl. Foliar segments produced clusters of nodular masses when cultured in MS/2 medium supplemented with 2.4-D and TDZ. These masses produced embryos. At first small and white, these embryos germinated and underwent consecutive stages of development. [22] obtained protocorms of *Aerides maculosa* Lindl. from the segment of young leaves grown *in vitro* in MS medium supplemented with 2 mg.L⁻¹ of BAP. The sub cultivation of protocorms in MS medium without the addition of regulators allowed their differentiation into plantlets.

When the protocorms were exposed to light, after 210 days of *in vitro* culturing in the dark, they became green, and after their transfer to basal culture medium without regulators, roots and leaves formed, thus regenerating into new complete orchid plantlets. According to [8], orchid seeds contain an embryo which is extremely small, consisting of a few hundreds of cells. Its development takes place when the seeds are under the influence of mycorrhizal fungi or when cultured in a nutritive medium where protocorms are formed. These protocorms are at first white, then greenish, often with long rhizoids in their equatorial plans and develop slowly into plants.

Effect of sucrose and pH in the *in vitro* growth of protocorms

The data related to the number of leaves collected 180 days after the inoculation of protocorms proved there is no effect of sucrose concentration or different pH levels since there have been no significant differences among the different treatments (Table 2).

The results obtained differ from those found by [23] when studying the interaction of sucrose and pH levels during the *in vitro* culturing of *Nephrolepis biserrata* (Sw.) Schott [23] observed that the concentrations of 15 and 45 g.L⁻¹ of sucrose increased the length and diameter of the leaves, and that in the absence of sucrose, no matter the pH level, there was an inhibition of the regeneration of sprouts and leaves.

The different pH levels did not influence the number of roots formed, whereas the sucrose concentrations, which allowed the formation of a greater number of roots, were 15, 30, 45 and 60 g.L⁻¹. The absence of the carbohydrate made the plantlets present little rooting (Table 2) as obtained by [3]. Was pointed out that the rooting of sproutings of *Diuris longifolia* R. Br. was favored by the increase of the sucrose concentration to 40 g.L⁻¹ or by the addition of 0.05% of activated coal to the culture medium [24], while studying the *in vitro* growth and rooting of *Dendrobium nobile* Lindl. seedlings observed that the addition of sucrose in the culture medium did not influence the *in vitro* rooting of the plants [12].

The present study did not see an influence of the pH in the number of leaves and roots. Studies carried by [25] registered the significant effect of the pH in the formation of leaves and roots of *Cymbidium* hybrids. pH 5.3 showed to be superior to pH 6.3 in the total number of leaves and roots produced from the protocorms inoculated *in vitro*.

For length of the longest root, pH 5.0 was significant only in the presence of 15 g.L⁻¹ of sucrose in the medium (1.09 cm), being that the other pH levels did not appear to play any role. The best sucrose concentrations for such variable were 15, 30 and 45 g.L⁻¹. The kind of radicular system obtained from *in vitro* rooting also determines the success of the transplant, being the shortest roots the most suitable ones, once they present themselves in the phase of active growth, easing the acclimation of the plantlet [5].

The effect of pH in height of aerial part was not evident, however with the supplementation in the medium with 15 and 30 g.L⁻¹ of sucrose, greater development of the aerial part (0.51 and 0.50 cm respectively) was



observed. These results differ from the ones found by [12] with *Dendrobium nobile* in which 60 g.L⁻¹ of sucrose provided greater growth of the aerial part.

The highest weight values of fresh matter (0.013 g) were obtained when the protocorms were cultured in medium supplemented with 15 g.L⁻¹ of sucrose and pH 5.0 or 5.8. When in sucrose-free media or in media containing 45 or 60 g.L⁻¹, the pH did not significantly influence the highest weight values of fresh matter.

The results obtained differ from those found by [26] when studying the influence of carbohydrate concentration in vegetative growth and rooting of *in vitro* *Oncidium varicosum*, in which the concentration of 60 g.L⁻¹ of sucrose was the best rate for all the variables evaluated.

In vitro propagated plantlets need an external source of energy as they are basically heterotrophic in this phase, thus unable to find the favorable conditions to perform photosynthesis [27]. According to Smeekens [28], carbohydrates can provoke alterations in the genic expression which are similar to the ones provoked by hormones. The perception of the signaling of sugar by proteic sensors provokes a series of events at cell level, altering the genic expression and the enzymatic activities, affecting germination, vegetative growth, reproductive development and seed formation. The transfer of the plantlets cultured with 30 g.L⁻¹ of sucrose to *ex vitro* acclimation conditions, in trays containing coconut powder as medium enabled the survival of 87.6% of the plants after 60 days of cultivation.

The association of 3 mg.L⁻¹ of 2,4-D and 1 and 3 mg.L⁻¹ of BAP provides the best formation of protocorms from radicular apices and foliar segments of *M. spectabilis* Lindl. var. *moreliana* Henfr. The concentrations of 15 and 30 g.L⁻¹ of sucrose, independently of the pH, appeared to be more suitable for the *in vitro* culture of the species, as they provided the formation of a larger number and length of the roots, height of the aerial part and weight of the fresh matter. pH appears to be significant only when combined with 15 g.L⁻¹ sucrose for variables of length of longest root and weight of the fresh matter.

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Table 1 – Effect of 2.4D and of BAP on the regeneration of protocorms from radicular apices and foliar segments of *Miltonia spectabilis* Lindl. var. *moreliana* Henfr. After 210 days of *in vitro* culturing (average of 30 explants). The maximum values obtained are in parentheses.

| Concentration (mg.L ⁻¹) | | Radicular apices | | Foliar segments | |
|-------------------------------------|----------------|------------------|----------------|-----------------|----------------|
| % explants | Average number | % explants | Average number | % explants | Average number |
| 2.4-D | BAP | with | of protocorms | with | of protocorms |
| protocorms | per explants | protocorms | per explants | protocorms | per explants |
| 0 | 0 | 0 | 0 | 3.1 | 0.003 (1) |
| 0 | 1 | 0 | 0 | 0 | 0 |
| 0 | 3 | 0 | 0 | 0 | 0 |
| 3 | 0 | 5.4 | 1.1 (34) | 0 | 0 |
| 3 | 1 | 5.4 | 2.4 (74) | 3.1 | 7.4 (221) |
| 3 | 3 | 2.6 | 5.6 (205) | 3.1 | 2.3 (77) |
| 30 | 0 | 0 | 0 | 0 | 0 |
| 30 | 1 | 0 | 0 | 0 | 0 |
| 30 | 3 | 0 | 0 | 0 | 0 |

Table 2 – Average values of leaves number (LN), roots number (RN), length of longest root (LR), height of aerial portion (HAP) and weight of fresh matter (FM) of plantlets of *Miltonia spectabilis* Lindl. var. *moreliana* Henfr. After 180 days of culturing in MS/2 medium with different concentrations of sucrose and pH levels.

| | Concentration (g.L ⁻¹) | | | | |
|---------------|------------------------------------|----------|----------|----------|----------|
| | 0 | 15 | 30 | 45 | 60 |
| | LN | | | | |
| pH 5.0 | 3.09 Aa | 3.76 Aa | 3.53 Aa | 3.16 Aa | 2.65 Aa |
| pH 5.8 | 2.74 Aa | 3.64 Aa | 3.52 Aa | 3.18 Aa | 2.73 Aa |
| pH 6.6 | 3.68 Aa | 3.57Aa | 3.03 Aa | 2.74 Aa | 2.67 Aa |
| | RN | | | | |
| pH 5.0 | 0.54 Aa | 2.18 Ab | 2.31 Ab | 2.16 Ab | 1.95 Ab |
| pH 5.8 | 0.52 Aa | 2.14 Ab | 2.32 Ab | 2.31 Ab | 2.01 Ab |
| pH 6.6 | 0.63 Aa | 1.93 Ab | 1.97 Ab | 2.14 Ab | 1.88 Ab |
| | LR (cm) | | | | |
| pH 5.0 | 0.25 Aa | 1.07 Bc | 0.96 Ac | 0.66 Ab | 0.66 Ab |
| pH 5.8 | 0.22 Aa | 0.75 Ac | 1.02 Ad | 0.72 Ac | 0.48 Ab |
| pH 6.6 | 0.28 Aa | 0.69 Ab | 0.78 Ab | 0.75 Ab | 0.34 Aa |
| | HAP (cm) | | | | |
| pH 5.0 | 0.38 Aa | 0.49 Ab | 0.45 Ab | 0.31 Aa | 0.31 Aa |
| pH 5.8 | 0.35 Aa | 0.47 Ab | 0.48 Ab | 0.38 Aa | 0.32 Aa |
| pH 6.6 | 0.39 Aa | 0.43 Aa | 0.35 Aa | 0.33 Aa | 0.29 Aa |
| | FM (g) | | | | |
| pH 5.0 | 0.007 Aa | 0.013 Bb | 0.009 Ab | 0.005 Aa | 0.005 Aa |
| pH 5.8 | 0.005 Aa | 0.013 Bb | 0.014 Ab | 0.007 Aa | 0.005 Aa |
| pH 6.6 | 0.008 Aa | 0.007 Aa | 0.010 Aa | 0.005 Aa | 0.005 Aa |

Averages followed by the same capital letter in the vertical line and low case in the horizontal line do not differ among themselves by the Skott-Knott test, at 5% of probability.