Plant Tissue Culture of Laelia Species

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Laelia orchids are cultivated by their splendid flowers and also widely used in orchid breeding. Plant tissue culture (PTC), defined as the aseptic culture of cells, organs, and their components under controlled *in vitro* conditions, is a biotechnological tool that has been successfully applied to recovery, conservation and clonal propagation of plants.

Laelia orchids | Neotropics | plant tissue culture | in vitro | Conservation | clonal propagation

1. Asymbiotic Seed Germination

In orchids, thin and non-endospermic seeds limit sexual reproduction [1][2]. In nature, it is calculated that out of one million seeds, only 10 to 15 germinate, and a maximum of two seedlings turn into adult plants [3]. Asymbiotic germination under in vitro culture conditions has been used for eight *Laelia* species thus far, showing this to be an effective alternative to increased germination and survival rates while maintaining genetic diversity. Furthermore, asymbiotic germination is a powerful technique for establishing aseptic cultures for other in vitro downstream purposes [4][5][6][7].

Two crucial factors for in vitro germination, capsule age and culture media composition, identified in all the protocols analyzed, are discussed as follows. Since almost all orchid species have dehiscent dry capsules [8], choosing capsules with closed valves that ensure no contamination but with already viable seeds is a challenge. Conversely, the chemical composition of culture media may determine if these viable seeds can germinate or not. To assess timing of collecting *Laelia* capsules, the most common strategy is testing capsules collected at different weeks after pollination (WAP) since capsule maturity positively controls seed viability, as reported recently [9]. For *L. albida*, *L. anceps* ssp. *dawsonii*, *L. marginata*, *L. purpurata*, and *L. rubescens*, the highest germination rates have been found in seeds from full-ripe capsules [10][11][12][9][13] or from close to this stage, namely 9 to 12 WAP. For *L. speciosa*, contrasting data have been reported. For example, Lavrentyeva and Ivannikov [14] found better results using seeds from immature fruits, while Ávila-Díaz et al. [15] indicated that higher rates of germination were found in seeds from older capsules (9 WAP). These last researchers also indicated that germination can be promoted in immature seeds if basal media are supplemented with 6-benzylaminopurine (BAP) at 2.2 µM and darkness, but the development of protocorms was not observed [15].

Knudson C (KC) medium [16] and Murashige and Skoog (MS) medium [17] have been the most frequent basal media tested for asymbiotic germination of *Laelia* species. KC medium has been evaluated for *L. albida*, *L. anceps*, and *L. purpurata* [10][14][12], while MS medium has been found adequate for *L. autumnalis*, *L. marginata*, *L. rubescens*, and *L. tenebrosa* [4][18][19][13]. These two media, KC and MS, have different nitrogen (N) contents; in KC medium, N is found at 16.04 mM, whereas in MS medium, N is found at 60.01 mM [20]. High N concentrations have an inhibitory effect on asymbiotic germination, but its magnitude seems to be species-dependent [20][21][22]. For example, KC inhibits seed germination completely in *L. rubescens* [9] but yields germination rates ranging from 70% to 90% in *L. albida* [10]. In basal media with low N concentration or used at diluted concentrations, e.g., Phytamax or 1/2 MS, peptone is usually added as an extra N or amino acid source [20][22][23][24]. Peptone addition has been assayed in *L. anceps* and *L. rubescens*, yielding high germination rates [14][9]. In other *Laelia* species such as *L. albida* and *L. purpurata*, organic N has been supplemented using potato starch [10] or ripe banana pulp [12], respectively.

2. Callus Culture

The callus, defined as a mass of proliferating and pluripotent cells [25], has been used for plant regeneration and clonal propagation, but if kept undifferentiated, it also represents an easy and scalable way to produce secondary metabolites for pharmaceutical purposes [26][27]. Callus formation involves cellular undifferentiation and a high cell division rate, which depend on explant type, genotype, culture medium composition, hormone type, and concentration [28][29]. Although callus culture is a commonly used PTC technique in high market-valued genera such as *Phalaenopsis*, *Dendrobium*, *Vanda*, and *Oncidium* [30][31][32], in *Laelia* orchids, it has only been tested in *L. anceps* ssp. *dawsonii* and *L. speciosa*, using seeds or leaf-like initial explants, respectively [26][11]. For *L. anceps*, friable calli were obtained from seeds germinated in MS medium supplemented with 2 $mg{\cdot}L^{-1}$ of 1-napthaleneacetic acid (NAA), BAP, and indole-3-acetic acid (IAA), 45 days after sowing [11]. In *L. speciosa*, callus induction was only observed when 6-month-old leaf segments of in vitro plantlets were used as initial explants. After 60 days of culture, light-green colored calli were obtained using 2.5 $mg{\cdot}L^{-1}$ BAP as the best treatment [26]. In both *Laelia* species, callus culture was used as tool for mass propagation via subsequent SE (*L. anceps*) or protocorm-like bodies (PLBs) regeneration and development (*L. speciosa*) [26][11].

3. In Vitro Conservation

Besides plant propagation and genetic improvement, in vitro PTC has been used as a tool for short-, medium-, and long-term storage of elite or critically endangered germplasm, by using technologies that imply encapsulation/cryoconservation or slow-growth conservation of several propagules, namely seeds, embryos, apical and axillary buds, microshoots, nodal segments, microplantlets, calli, and PLBs [33][34][35][36]. During cryoconservation, plant material is stored in liquid nitrogen (LN) (−196 °C) or in the vapor phase (from −150 to −196 °C) [37]. By using seeds or pollen, significant advances have been reported in cryoconservation for the genera *Dendrobium*, *Phalaenopsis*, *Cymbidium,* and *Cattleya* [37][38]. In the case of orchid seeds used for ultra-low freezing purposes, desiccation and use of plant vitrification solutions (PVS) as cryoprotectans are key pre-treatments for

keeping viability [37][39]. In *Laelias*, seed cryoconservation has only been assayed in *L. autumnalis* and *L. speciosa* thus far [40][41]. For these species, the best treatments for seed desiccation implicate air-drying and silica gel (sodium silicate). In *L. speciosa* seeds, the use of PVS2 (glycerol 30% + ethylene glycol 15% + dimethylsulfoxide + 0.4 M sucrose) or PVS3 (glycerol 50% + sucrose 50% at 0.4 M) negatively affects seed viability and asymbiotic germination [41], contrary to reports for PLBs in other orchid species [42][43][44]. Recently, PVS2 was used successfully in *Encyclia cordigera*, keeping seed viability at 93.79% after LN exposure [45]. Assays with PSV2 and PVS3 in *Laelia* species are preliminary, and therefore, evaluation of different exposure times to these solutions and the use of other cryoprotectant agents commonly used in vitro cryoconservation, namely dimethylsulfoxide, ethylene glycol, glycerol, and sucrose [46], have to be tested. These results will be key factors during the development of vitrification-based cryopreservation protocols for *Laelia* orchids.

Another strategy for in vitro conservation during short to medium-long periods (few months to 2–3 years) is slow growth without subculturing [47]. A combination of 7.4 µM BAP and 5.3 µM NAA has been suggested as adequate for conservation of PLBs of *L. albida* [10], but a detailed protocol is still needed. In contrast, a full protocol using asymbiotic germination-derived plantlets that allows in vitro conservation and regeneration was reported for *L. anceps* recently. Paclobutrazol at 2 $mg \cdot L^{-1}$ is an effective treatment for producing reduced growth of shoots and roots with a 90% survival rate [48].

4. PLBs Proliferation

In the PTC of orchids, one of the most interesting processes observed under specific conditions is the formation, proliferation, and regeneration of PLBs. These explants are excellent for mass multiplication of orchids in semisolid media and bioreactor systems. Currently, PLBs are used as models to dissect genetic circuits involved in regeneration and metabolite production in orchids under in vitro conditions [30][49][50][51]. Although PLB formation has been observed in many *Laelia* species, especially in those germinated asymbiotically [26][9], few protocols for enhancing their in vitro proliferation have been reported thus far. In *L. speciosa*, PLB proliferation was obtained after 60 days of subculture of leaf-derived calli in a MS medium supplemented with 2.5 $mg \cdot L^{-1}$ NAA and 1 $mg \cdot L^{-1}$ BAP [26]. Lavrentyeva and Ivannikov [14] reported a single medium for PLB proliferation for *L. anceps*, *L. lobata*, *L. lundii*, *L. mantiqueirae*, *L. purpurata*, *L. rubescens*, and *L. sincorana*: basal buds of shoots in a MS medium supplemented with 5 $mg \cdot L^{-1}$ BAP, 2 $mg \cdot L^{-1}$ NAA, 100 $mL \cdot L^{-1}$ peptone, 15% coconut milk, and 1.5 $g \cdot L^{-1}$ activated charcoal. This protocol should be revised and optimized for members of the *Laelia* genus, as it is long known that a morphogenic response to in vitro culture is a species-dependent process. In *L. superbiens*, PLB formation has been observed in shoots after 30 days of incubation with thidiazuron and meta-topolin, both at 0.44 µM; however, this effect has not been quantified [52].

5. Organogenesis-Mediated Regeneration

Organogenesis has been assayed in three *Laelia* species: *L. anceps*, *L. gouldiana*, and *L. superbiens*. In all of them, the most responsive initial explants were those obtained from plantlets previously germinated in vitro but not

those collected from ex vitro mature plants [53][52][54]. In *L. anceps*, leaves collected from in vitro plantlets were used as initial explants, and after 90 days, a MS medium complemented with 10 mg·L^{-1} BAP and 5 mg·L^{-1} KNO_3 yielded the highest rate of PLB formation [53]. Similarly, leaf segments of *L. gouldiana* have been used to regenerate shoots; in this case, the medium consisted of MS salts and 0.1 mg·L^{-1} NAA and 45–75 days of induction [54]. Temporary immersion bioreactors have also been used to establish organogenesis in *Laelia* species. The RITA® system was tested in *L. superbiens* to obtain shoots directly from plantlets without an intermediate callus phase or PLBs [47]. In this case, although the combination RITA® bioreactor + half strength MS enriched with 100 mL·L^{-1} coconut water was more efficient for enhancing shoot biomass than other assayed treatments, a higher number of shoots were quantified in semisolid media + full strength MS containing 100 mL·L^{-1} coconut water. In both treatments, these effects were positively affected by using 0.44 µM BAP and 1 mg·L^{-1} chitosan [52].

6. Somatic Embryogenesis

Having considered the forefront of PTC because of its capacity to provide a large number of plants in a shorter time than organogenic approaches [55], SE is one of the most important techniques for mass propagation of orchids [56]. Paradoxically, in the *Laelia* genus, SE has been little explored, and only *L. anceps* ssp. *dawsonii* has been tested for SE response. In this species, indirect SE has been obtained from callus derived from asymbiotically germinated seeds. Optimum induction conditions for embryogenic calli consisted of MS supplemented with NAA, BAP, kinetin and IAA (2 mg·L^{-1} each) [57][11]. The reports indicate that kinetin may be excluded, and the induction medium still works efficiently [58]. Growth of germinated somatic embryos was promoted by using a Vacint and Went (VW) medium, containing 2 mg·L^{-1} BAP, 1 mg·L^{-1} IAA and 0.2% activated charcoal [58].

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