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Filter paper inhibits *in vitro* protocorm-like body formation in hybrid *Cymbidium* and reduces synseed germination, but buffers the negative impact of antibiotics

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Abstract: Only few studies in the plant tissue culture literature have examined the impact of filter paper on *in vitro* plant organogenesis. In this study, using a model plant, hybrid *Cymbidium* Twilight Moon 'Day Light', the impact of a single or double layer of Advantec #2 or Whatman #1 filter paper on new protocorm-like body (*neo*-PLB) formation on Teixeira *Cymbidium* (TC) medium was examined for half-PLBs (transgenic and non-transgenic), PLB-derived transverse thin cell layers (tTCLs), and PLB synseeds. In addition, the response of half-PLBs or tTCLs to two antibiotics (kanamycin and cefotaxime, commonly used in plant genetic transformation studies) was investigated either directly on gelled medium or on filter paper-overlaid medium. Filter paper negatively affected most growth and developmental parameters of all the explants tested, both transgenic and non-transgenic. A double sheet of filter paper had a significantly ($P \leq 0.05$) more negative impact than a single sheet, relative to the control values (i.e., no filter paper). Kanamycin inhibited *neo*-PLB formation on TC medium, the negative impact being greater on a single layer than on a double layer of filter paper, i.e., filter paper buffered the growth-inhibiting characteristics of kanamycin. Up to 100 mg/l, cefotaxime showed no apparent negative effects on *neo*-PLBs formation and growth, although hyperhydricity was observed when filter paper was not used.

Keywords: antibiotics, cefotaxime, genetic transformation, kanamycin, orchid, protocorm-like body, Teixeira *Cymbidium* (TC) medium

INTRODUCTION

To date, only one study has examined the impact of filter paper on plant organogenesis *in vitro* (Teixeira da Silva 2003). In that landmark study, the following important findings were reported: (1) Advantec #2 and Whatman #1 filter paper improved organogenesis in 2 chrysanthemum cultivars; (2) Advantec #2, Whatman #1 and Whatman #3 filter paper inhibited tobacco embryogenesis and explant survival; (3) any of these three filter paper types reduced the negative impact of antibiotics

(kanamycin or cefotaxime) by as much as 50%. That study was important because many plant tissue culture scientists plate their explants onto filter paper, for example after genetic transformation, while many others plate their explants directly onto agarized medium. This study, based on the Teixeira da Silva (2003) study, departed from the hypothesis that filter paper can affect plant organogenesis (*sensu lato*). More recently, it was shown that filter paper bridges can negatively impact hybrid *Cymbidium* plantlet growth in liquid medium (Teixeira da Silva 2013c). In orchid biotechnology, many issues pertaining to *in vitro* responses have not been studied and there is a requirement to fill that gap in the literature with basic, yet fundamental studies that test such responses (Hossain et al. 2013; Teixeira da Silva 2013a). Using standard explants for orchid growth *in vitro*, somatic embryos or protocorm-like bodies (PLBs) as well as PLB-derived transverse thin cell layers (tTCLs), this study aimed to examine how such explants respond to optimized medium if grown directly on agarized medium or if that medium was overlaid with a single or double layer of filter paper. The study used hybrid *Cymbidium*, which has emerged as a new model plant due to its well-characterized *in vitro* growth (Teixeira da Silva & Tanaka 2006; Teixeira da Silva 2013b; Teixeira da Silva & Dobránszki 2013a). Moreover, TCLs, which have been shown to be extremely sensitive explants for *in vitro* regeneration studies in orchids and dozens of other plant families (Teixeira da Silva 2013b; Teixeira da Silva & Dobránszki 2013b) were used. The study also examined the developmental response of synthetic seeds, or synseeds, to filter paper, as they are useful vessels for storage of plant tissues in the short- to long-term, for example in cryopreservation (Teixeira da Silva 2013a; Sharma et al. 2013). Finally, the presence of antibiotics in the medium tends to reduce organogenesis in plant tissues, such as in tobacco or chrysanthemum (Teixeira da Silva et al. 2003), citrus (Eed et al. 2010), or strawberry (Qin et al. 2011, and references therein). However, antibiotics continue to be major selective agents for plant transformation studies, and thus the basic response of untransformed tissues should be studied prior to or simultaneously with the study of the response of transformed tissues. Consequently, it was of interest to examine how transformed and untransformed PLBs would respond to different antibiotics, at different concentrations, in the presence of filter paper. Two antibiotics were compared: kanamycin, a selective agent for plant tissues containing the *npt*II gene, and cefotaxime, which is used to control the growth of *Agrobacterium* spp.

MATERIALS AND METHODS

All protocols (experimental design, chemicals, reagents, explant preparation, and treatment analysis) follow 2012 and 2013 studies by Teixeira da Silva, almost *verbatim* in parts.

Chemicals and reagents

All chemicals and reagents were of the highest analytical grade available and were purchased from Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), the cheapest choice at the highest tissue-culture grade, unless specified otherwise.

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Tokushima, Japan), originally developed from shoot-tip culture on (Vacin & Went 1949) (VW) agar medium without plant growth regulators, were induced and subcultured (PLB induction and proliferation medium) every two months on Teixeira *Cymbidium* (TC) No. 1 medium (Teixeira da Silva 2012b), supplemented with 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin (Kin), 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA), following Teixeira da Silva et al. (2005) and Teixeira da Silva & Tanaka (2006). TC medium was used in this study even though several basal media can support the induction and development of *Cymbidium* PLBs *in vitro* (Teixeira da Silva et al. 2005). All media were adjusted to pH 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 kPa for 17 min. Cultures were kept on 40 ml of medium in 100-ml Erlenmeyer flasks, double-capped with aluminium foil, at 25°C, under a 16-h photoperiod with a light intensity of 45 μmol m^{-2} s^{-1}, provided by 40-W plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally dissected as two pieces of PLB segments, 3-4 mm in diameter and 10/flask, were used as explants for PLB induction and proliferation. Culture conditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva et al. 2005), biotic (Teixeira da Silva et al. 2006b) and abiotic factors (Teixeira da Silva et al. 2006a) for PLB induction, formation, and proliferation.

Cymbidium *explants used in trials*

All trials, detailed in the next sections, used standard half-PLBs and tTCLs, whose preparation is detailed in Teixeira da Silva (2013b). In addition, transgenic PLBs derived from particle bombardment with pWI-GUS (Teixeira da Silva & Tanaka 2009, 2011), and containing the *npt*II gene, were tested and maintained on selective medium containing 50 mg/l of kanamycin sulphate. Finally, encapsulated half-PLBs, or synseeds, were used, as prepared by Teixeira da Silva (2012a).

Response of control and transgenic Cymbidium *half-PLBs, tTCLs and synseed, to filter paper*

In trial 1, 10 half-PLBs (untransformed) or tTCLs were plated per Petri dish (90 mm diameter, 15 mm deep; AsOne, Osaka, Japan), cut surface down (half-PLBs) or basal side down (tTCLs), either directly on gelled TC medium or on top of a single or double layer of filter paper. Two makes of filter paper were tested: Advantec #2 or Whatman #1. In the same way, 10 synseeds in trial 2 and 10 transgenic half-PLBs in trial 3 were plated, like in trial 1.

Response of control and transgenic Cymbidium *half-PLBs to antibiotics*

Half-PLBs were plated directly on gelled TC medium or on top of a single layer of Advantec #2 filter paper. All media contained the following concentrations of antibiotics: 0 (control), 25, 50, or 100 mg/l of kanamycin sulphate or 50, 100 or 500 mg/l of cefotaxime, based on Teixeira da Silva et al. (2003) and using the experimental design of Teixeira da Silva (2012b). All antibiotics were only added to solid TC medium after cooling at room temperature and filtering through 22 μm Millipore filters.

Statistical analyses

Experiments were organized according to a randomized complete block design with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate (n = 30, total sample size per treatment). Data were subjected to analysis of variance (ANOVA) with mean separation by Duncan's multiple range test using SAS® version 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leq 0.05$.

RESULTS AND DISCUSSION

The most notable finding of this study is that filter paper decreased most growth and developmental parameters of all explants tested, transgenic and non-transgenic, with the double sheet having a significantly more negative impact than the single sheet, relative to the control values (i.e., no filter paper) (Fig. 1). This study indicates that filter paper has a negative impact on *Cymbidium in vitro* growth. Interestingly, a similar trend was observed for tobacco and chrysanthemum (Teixeira da Silva 2003). Regrettably, no other study in the literature exists on the impact of filter paper on plant organogenesis. It is conceivable that filter paper may absorb moisture from the medium and inhibit the flow of nutrients from agarized medium to the explant, causing the explant to experience desiccation stress or reduced access to humidity and nutrients essential for growth. This hypothesis is supported by the fact that when a single layer of filter paper, either Advantec or Whatman, was placed on the surface of agarized TC medium, most parameters in Fig. 1 dropped significantly, and even more when a double layer was used. Excessive drying was already shown to negatively impact the germination of PLB synseed (Teixeira da Silva 2012a, 2013d), therefore the reduction in synseed-related parameters (Fig. 1) may also be a desiccation-related response.

When a single layer of filter paper was laid over TC medium, half-PLBs formed significantly fewer *neo*-PLBs than the control in the presence of kanamycin, and this negative effect increased as the concentration of kanamycin increased (Fig. 2). The negative effect was less pronounced when a double layer of filter paper was used, but was still worse than the control (i.e. no kanamycin), suggesting that filter paper buffers or reduces the negative impact on plant growth and development. A similar negative impact of aminoglycoside antibiotics was observed for chrysanthemum and tobacco (Teixeira da Silva et al. 2003), citrus (Eed et al. 2010) and strawberry (Qin et al. 2011).

Interestingly, although cefotaxime did not increase the number of *neo*-PLBs significantly more than the control, the number was significantly equal, suggesting that cefotaxime did not have any apparent negative effects on *neo*-PLBs formation and growth, at least up to 100 mg/l. However, fresh weight of explants + *neo*-PLBs increased significantly more than in the control (Fig. 2), and the hyperhydric nature of those explants suggests that cefotaxime somehow allowed the excessive absorption of water from the medium, even in the presence of filter paper. Cefotaxime has been shown to stimulate shoot production in tobacco and chrysanthemum (Teixeira da Silva et al. 2003), somatic embryogenesis in *Dianthus* (Nakano & Mii 1993), and

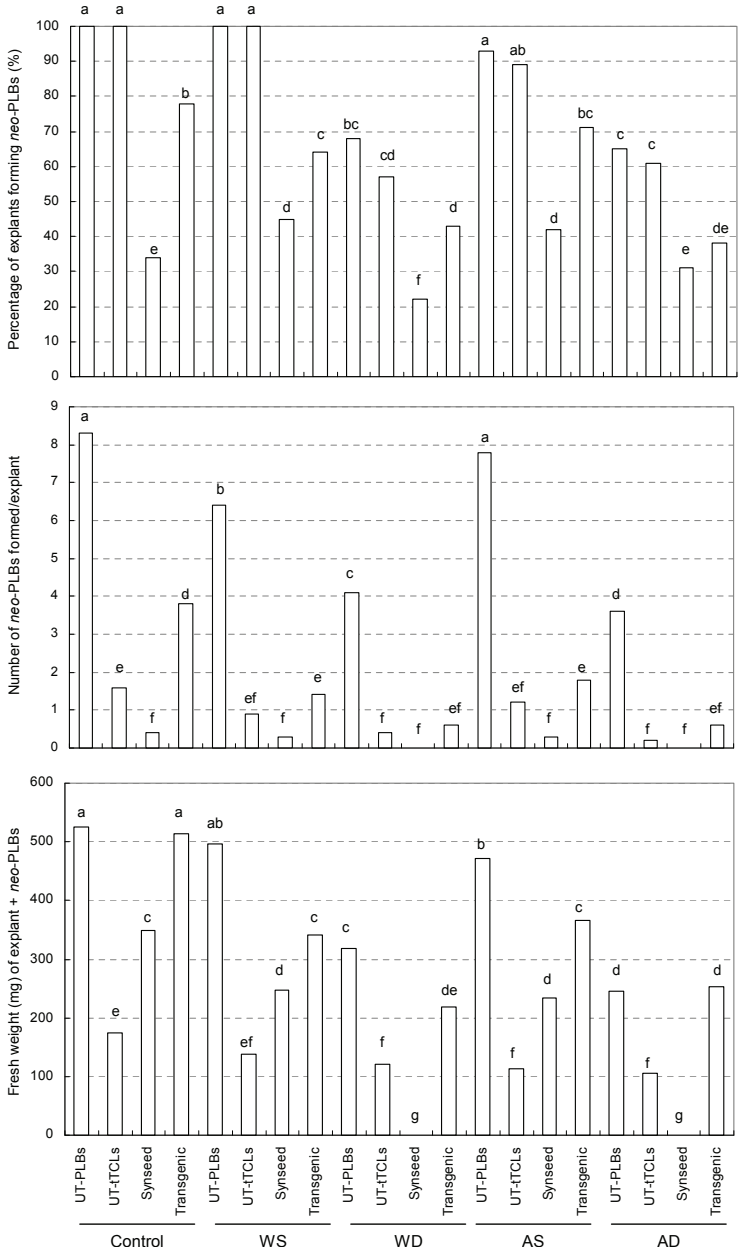

Fig. 1. Effect of filter paper on several explant types: growth and developmental responses to two types of filter paper (single or double sheet) or no filter paper (control, i.e., explant plated directly on agar), determined after 90 days of culture on the medium. A = Advantec filter paper; D = double sheet; PLB = protocorm-like body; S = single sheet; UT = untransformed; W = Whatman #2 filter paper. Mean values marked with the same letters for the same parameter are not significantly different based on Duncan's multiple range test ($P \leq 0.05$), $n = 90$ ($10 \times 3 \times 3$)

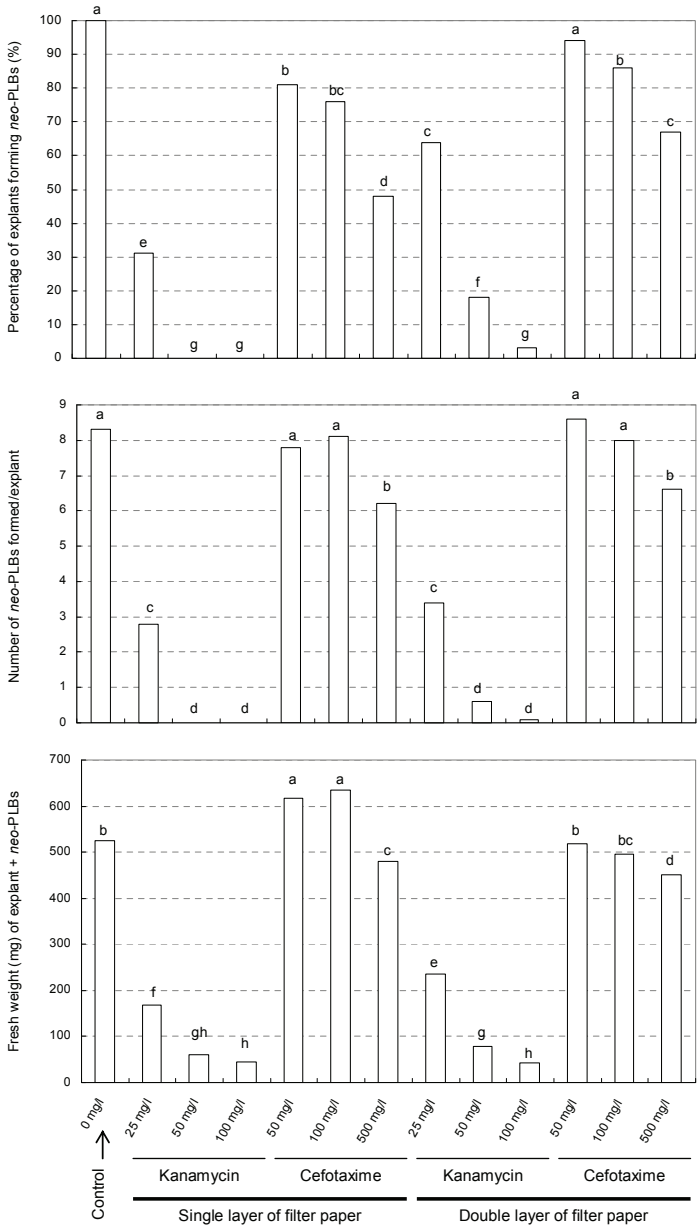

Fig. 2. Effect of 2 antibiotics (kanamycin-sulphate and cefotaxime) on half-PLBs in the presence of a single or double layer of Advantec filter paper, or no filter paper (control: explant plated directly on agar): growth and developmental responses determined after 90 days of culture on the medium. PLB = protocorm-like body. Mean values marked with the same letters for the same parameter are not significantly different based on Duncan's multiple range test ($P \leq 0.05$), $n = 90$ ($10 \times 3 \times 3$)

shoot development in pine cotyledons (Humara & Ordás 1999). Cefotaxime is broken down into the auxin phenylacetic acid (Holford & Newbury 1992), explaining why it would stimulate growth of *in vitro* cultures.

Even though filter paper is commonly used as a base for plating explants on a solid medium, none of those studies has assessed the impact of plating the same explants directly on filter paper-free medium. Moreover, filter paper is a commonly essential component of paper bridges that allow explants to develop, with the filter paper acting as a wick to absorb liquid medium, for example in test tubes. Filter paper is also often used in screening for plant extracts and metabolites and their antibacterial, or other biological (e.g., allelopathic) qualities. Finally, filter paper is almost always used in seed germination experiments and serves as a base. Examples of these 4 cases of the use of filter paper are not covered in this paper. Experiments with chrysanthemum, tobacco and *Cymbidium* indicate that repeated sub-culture onto solid medium overlaid with filter paper can reduce the incidence of browning (J. A. Teixeira da Silva, unpublished data). In contrast, the literature on the impact of antibiotics on *in vitro* growth of plants is much better documented (e.g. Padilla & Burgos 2010), most likely because antibiotics are used for genetic transformation systems and also to control bacterial and fungal infections.

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