*Effect of phyllotactic position and cultural treatments toward successful direct shoot organogenesis in dwarf 'Pixie' grapevine (*Vitis vinifera *L.)*

Kathie L. Nicholson, Nathan Tarlyn, Tyler Armour, Mark E. Swanson & Amit Dhingra

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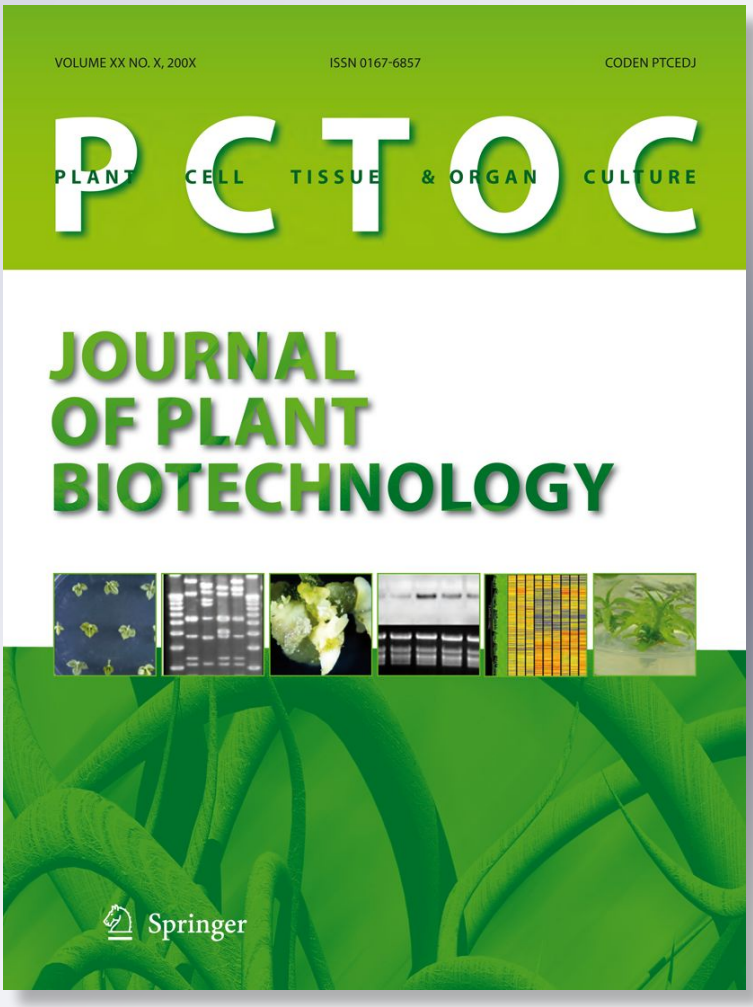

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RESEARCH NOTE

Effect of phyllotactic position and cultural treatments toward successful direct shoot organogenesis in dwarf 'Pixie' grapevine (*Vitis vinifera* L.)

Kathie L. Nicholson · Nathan Tarlyn · Tyler Armour · Mark E. Swanson · Amit Dhingra

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Abstract In *Vitis* spp. where somatic embryogenesis-based regeneration predominates, an efficient, reproducible and robust method of direct shoot organogenesis from leaf explant material has been established in the dwarf wine grape 'Pixie' (*Vitis vinifera*). This regeneration system was achieved by testing the response of leaf material in two stages of development, and pre-conditioning the explant material in dark conditions and/or in liquid media prior to excising from the plant and placing it on solidified media. The pre-excision treatments included (1) a dark period of 24 h, with no regeneration medium; (2) soaking in regeneration medium followed by a dark period of 24 h; (3) a dark period of 24 h followed by soaking in liquid VRM (*Vitis* Regeneration Medium); (4) vacuum infiltration in liquid VRM followed by a dark period of 24 h; and (5) a control of no pre-conditioning treatment. Excised leaves from pre-treated intact plants in vitro significantly increased the frequency of shoot organogenesis. The most responsive explant material consisted of young semi-translucent apical leaves varying in size from 3 to 8 mm in length. The most successful combinations of factors contributing to shoot organogenesis involved the solely dark-exposed apical leaves or the soaking in VRM followed by a dark period. These results are expected to facilitate *Vitis*-related research in genetics, functional genomics, physiology, and other fields.

K. L. Nicholson · N. Tarlyn · T. Armour · A. Dhingra (✉)
Department of Horticulture, Washington State University, Pullman, WA 99164, USA
e-mail: adhingra@wsu.edu

M. E. Swanson
Department of Natural Resource Sciences, Washington State University, Pullman, WA 99164, USA

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Introduction

Grapevine (genus *Vitis)* is an economically important perennial crop representing a world-wide international commodity value of over $39 billion in 2010 (FAOStat 2012). Global wine production in 2010 was 4.4 billion liters (FAS/USDA 2011), the majority of which was produced from the European wine grape (*Vitis vinifera* L.). In the United States, grapes are the most important non-citrus fruit crop by tons produced and value, with wine grapes representing approximately 66 % of the total value of all grape production, the other 34 % being table, raisin, and juice grapes (FAOStat 2010). Over the last few years, great strides have been made in generating genomic resources for grapevine (Jaillon et al. 2007 and Zharkikh et al. 2008). However, its perennial habit has impeded the application of reverse genetics approaches to understand gene-trait relationships in *Vitis* spp.

As a potential model system for functional genomics research in *Vitis* spp., the Pixie grapevine, a continuously flowering dwarf variety developed from Pinot Meunier, was released in 2006 by the USDA Agricultural Research Service (Cousins and Tricoli 2006). Pinot Meunier is a periclinal chimera with separate mutations in the L1 and L2 layers which independently exhibit different phenotypes (Franks et al. 2002). The L1 layer contains a mutation that causes gibberellin insensitivity and is responsible for the dwarf characteristics of Pixie (Boss and Thomas 2002; Cousins and Tricoli 2006), making Pixie ideal for research. Since maintenance of Pixie in the greenhouse and in the

limited space of aseptic tissue culture is unproblematic compared to standard *V. vinifera* varieties, Pixie is considered to be a potential research model for all species of *Vitis*. The standard varieties demand a large amount of greenhouse space, constant pruning, and require frequent transfer in tissue culture. During the 5 years we have been growing Pixie in the greenhouse, we have observed that 4–6 Pixie vines can be grown in the space required for one standard vine, and pruning of Pixie is necessary only 2 or 3 times a year, compared to 1 or 2 times a month for standard varieties. Additionally, we have found that Pixie survives 6–8 months in tissue culture without transfer, while standard varieties outgrow their space in 1–1/2 to 2 months and must be pruned and transferred.

Currently there is much less focus on developing new wine grape cultivars compared with other fruit crops, with little interest in transgenic grapevines. However, an efficient and rapid method for grapevine in vitro regeneration via organogenesis is an invaluable resource for true-to-type propagation, nuclear and plastid transformation (Dhingra and Daniell 2006), and will facilitate functional genomics efforts toward understanding the grape genome. In vitro regeneration of *V. vinifera* from explant material has proven to be difficult, and success has been reported to be dependent upon specific genotypes within the species (Maillot et al. 2006; Martinelli et al. 1996; Peros et al. 1998). Peros et al. (1998) observed that Pinot Noir and Pinot Meunier had among the lowest capabilities for organogenesis among all the cultivars they tested. Therefore, development of a successful protocol for direct shoot organogenesis of Pixie, a descendent of those two varieties, will have far-reaching implications for successful regeneration of other recalcitrant cultivars.

There are reports of limited success toward the development of direct shoot organogenesis of *V. vinifera* (Colby et al. 1991; Mezzetti et al. 2002; Peros et al. 1998; Stamp et al. 1990a, b; Torregrosa and Bouquet 1996). An early study of direct shoot organogenesis (Colby et al. 1991) suggested that transformed leaf lamina cells exhibiting GUS protein (beta-glucuronidase gene) expression were never involved in shoot regeneration. As a result, it was concluded that direct shoot organogenesis was an ineffective method for transformation. At the present time, somatic embryogenesis has become the most accepted regeneration method for the purposes of propagation and transformation (Bouquet et al. 2006; Das et al. 2002; Dhekney et al. 2009; Jaskani et al. 2008; Li et al. 2008; Maillot et al. 2006; Mulwa et al. 2007). This is in contradiction of the general judgment that the products of callus-generated embryogenesis are not as genetically stable as those produced via direct organogenesis (D'Amato 1975). Additionally, somatic embryogenesis is a process that takes an extended amount of time in tissue culture, further predisposing the plant material to unwanted genetic rearrangements. Somatic embryogenesis is especially problematic for *V. vinifera*, since grapevines frequently exhibit periclinal chimerism (Hocquigny et al. 2004; Pelsy 2010; Stenkamp et al. 2009) and it has been shown that this genetic chimerism may not be preserved through somatic embryogenesis as it is through organogenesis (Bertsch et al. 2005). Preserving genetic chimerism is pertinent in case of *Vitis* to prevent unwanted genetic alterations in a variety during regeneration and transformation. Therefore, the development of a reliable protocol for direct shoot organogenesis of Pixie has the potential to contribute to a more stable process of regeneration for all standard grape varieties. The capability for direct shoot organogenesis in *V. vinifera* will have a positive, far-reaching impact on further grape research, ultimately providing positive input to the grape and wine industry worldwide. In this study, we tested a set of parameters at pre- and post-excision stages of the grapevine to identify optimal conditions for efficient shoot organogenesis.

Materials and methods

Plant materials

Material used for shoot organogenesis was from dwarf Pixie grapevine (*Vitis Vinifera* L.) originally obtained from David M. Tricoli, Ralph M. Parsons Foundation Plant Transformation Facility, University of California, Davis, CA. Plants were propagated and maintained both in the greenhouse in potting soil and in vitro on woody plant medium (WPM) consisting of Lloyd and McCown (1981) salts, vitamins, and iron, supplemented with 1 g/L casein hydrolysate, 195 mg/L MES (1 mM), 500 mg/L activated charcoal, 100 mg/L inositol, 20 g/L sucrose, 0.01 mg/L 1-naphthaleneacetic acid (NAA), solidified with 8 g/L agar (Bioworld), and pH adjusted to 5.6. Leaf explant material for direct shoot organogenesis was obtained by propagating nodal cuttings from axenic plants grown in vitro, nine nodal cuttings per magenta box on WPM. Leaf material was ready for use when plantlets had started to root and developed two to five leaves. Explant material consisted of the two most apical leaves of the shoot tip according to phyllotactic position: (1) Semi-transparent, not fully expanded apical leaves, referred to as age 0 leaves; and (2) Progressing basipetally, the next leaf just fully expanded measuring 10–15 mm in length at the next node down the shoot from the age 0 leaf, referred to as age 1 leaf (Fig. 1a, b). Leaf explants were excised from the plant retaining a section of petiole, but were not further wounded or cut before plating on the regeneration medium.

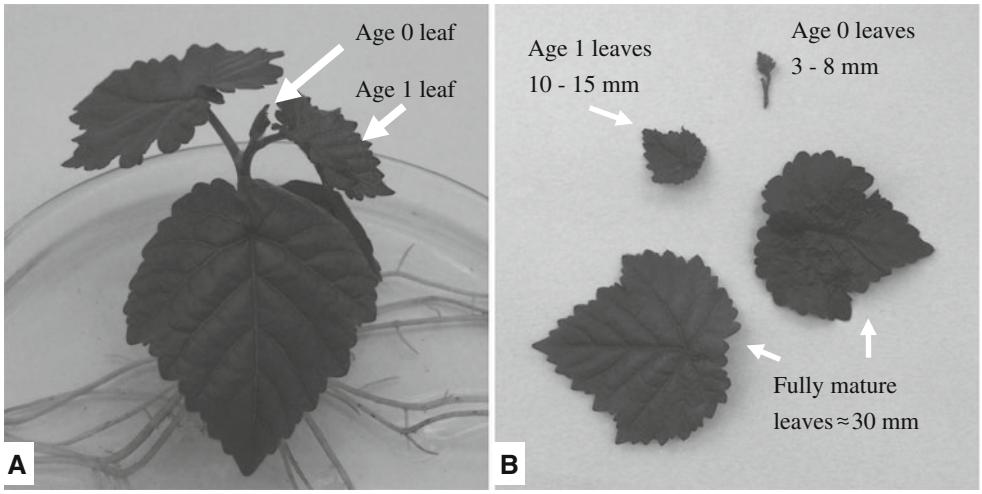

Fig. 1 Pixie plantlet illustrating position and size of age 0 and 1 leaves. **a** Intact plantlet in tissue culture and **b** Separated leaves with average size indicated in mm

Pre-conditioning treatments

Prior to excising leaf explants, the undisturbed plants were subjected to five pre-conditioning treatments. The treatments included: (1) a dark period of 24 h; (2) a 30 min soak in liquid *Vitis* regeneration medium (VRM: details regarding this media are found in the next section), followed by a dark period of 24 h; (3) a dark period of 24 h followed by a 30 min soak in liquid VRM; (4) a 30 min vacuum infiltration in liquid VRM followed by a dark period of 24 h; and (5) a control of no pre-conditioning treatment.

Shoot organogenesis

Following these treatments, apical leaves of different ages were used for the induction of shoot organogenesis. Leaves were excised from the plant, placed (abaxial surface down) on VRM consisting of MS (Murashige and Skoog 1962) salts and vitamins, N6 (Chu et al. 1975) micro salts with 16 g/L glucose, 100 mg/L inositol, 750 mg/L MES, 6.75 mg/L benzyladenine (BA), 0.75 mg/L thidiazuron (TDZ), 0.25 mg/L indole-3-butyric acid (IBA), and solidified with 5.6 g/L agar (Phytotechnology Plant TC Agar), and pH adjusted to 5.6. After plating, the explants were incubated in the dark for 14 days. After the 14 day dark period, the explants were transferred to *Vitis* development medium (VDM), which is identical to VRM but with an altered phytohormone content of 1 mg/L BA and 0.1 mg/L IBA. The explants were then moved to low light (15-30 μmol m^{-2} $s^{-}1$), where they remained until shoots developed 3–4 distinct internodes, and were ready to be excised from the explant and transferred to WPM for rooting.

Field emission scanning electron microscopy (FESEM)

Primordia that developed early on the petiole and leaf edges of mother explants were selected for microscopic analysis. Explants were directly imaged in a low vacuum mode on a FEI 200F field emission scanning electron microscope at the Franchesci Microscopy and Imaging Center (Washington State University, Pullman, WA). Imaging was performed using an accelerating voltage of 10 kV and a stage temperature of 1 °C.

Statistical analysis

The success of organogenesis was based on number of explants that formed regenerative masses versus those that did not (Fig. 2). Logistic regression, implemented in a generalized linear model framework (Fahrmeir and Tutz 1994; McCullagh and Nelder 1989) in the R environment for statistical programming (R Core Development Core Team 2008) was used to assess the effect of leaf age/size (unfolding apical leaves vs. the next expanded leaf) and treatments on successful organogenesis. Since the dependent variable was comprised of a binary outcome, logistic regression was selected as the appropriate model framework (Fahrmeir and Tutz 1994). The logistic regression model specification consisted of success vs failure of organogenesis as a function of leaf age + treatment + leaf age x treatment:

$$\mathrm{ORG} = \mathrm{f}(\mathrm{LA} + \mathrm{TRT} + \mathrm{LA} * \mathrm{TRT})$$

where ORG = success/failure of organogenesis, LA = leaf age, TRT = treatment. Wald tests employing specific orthogonal contrasts were used as a post hoc test to assess the effects of treatments as a group against the control.

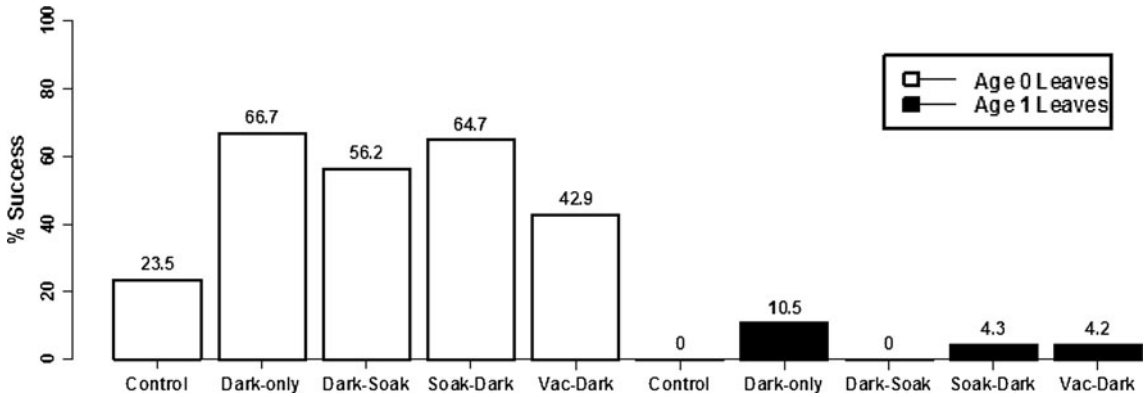

Fig. 2 Percent success (regeneration vs. no regeneration per explant) observed by age and treatment

Results and discussion

Identification of parameters for establishing organogenesis in Pixie

Prior to the protocol described in this research, five sequential experiments were conducted to test the optimal concentrations of BA, TDZ, and IBA for successful regeneration and the advantage of glucose over sucrose as the carbon source. In the first two experiments, sparse regeneration was observed and inhibitory browning of explants occurred following their transfer to low light. This possibly accounted for the lack of copious regeneration, as browning has been reported to interfere with in vitro regeneration of *Vitis* spp. and other plants (Dhavala and Rathore 2010; Stamp et al. 1990b). Although the explant material consisted of young leaves that were not as photosynthetically active as older leaves, it was hypothesized that sucrose in the medium was contributing to excessive stress and browning. High levels of sucrose are known to inhibit photosynthesis (Chen et al. 2005) and suppress photosynthetic gene function (Koch 1996), leading to plant stress. Additionally, tissue culture conditions in general contribute to overall stress of explant material (Cassells and Curry 2001). Such conditions are ideal for oxidative stress leading to the production of reactive oxygen species, and overproduction of phenolic compounds resulting in explant browning, inhibited regeneration, and eventually death. Replacing sucrose with glucose reduced the browning significantly in experiment 3 and contributed to increased regeneration capability in subsequent trials. In order to optimize the uptake of phytohormones from the medium, experiment 4 initiated a pre-treatment of excised leaf material in liquid medium. The soaking treatment caused excessive tissue death, but the explant material that survived exhibited a high rate of regeneration (Fig. 3a).

To overcome the stress to the leaf explant material of the pre-treatment soak in experiment 4, intact plants were covered for 30 min with liquid VRM in the magenta box. Additionally, the last experiment examined the regeneration capability of leaves at different stages of development. Through these initial experiments it was established that the most responsive explant material consisted of the smallest just expanding whole apical leaves. Following this observation, the focus of the research shifted towards comparing shoot regeneration between apical leaves of different ages (Fig. 1a) with relation to various cultural treatments.

Leaf age and cultural treatment determines organogenesis potential

Leaf age was found to be a significant contributing factor for the regenerative potential of leaves of Pixie grapevine. Age 0 leaves exhibited minimal callusing and browning, and direct shoot organogenesis occurred at various locations on the leaf lamina often in proximity to vascular tissue (Fig. 3b, c). FESEM revealed petiole and leaf edges as the organogenic sites where formation of shoot primordia occurred. Some of these primordia resembled that of a ruffle-shaped structure emerging from pleated plant tissues (Fig. 4a), while others were distinct shoot primordia emerging singly and in multiples from excised end of the age 0 leaf petiole (Fig. 4b, c) and the background organogenic callus tissue (Fig. 4d), respectively.

Age 0 leaves represent apical, just expanding or not fully expanded, semi-translucent leaves varying in size from 3 to 8 mm in length (Fig. 1b). These leaves are actively growing, photosynthate utilizing sinks and presumably more receptive to the uptake of applied phytohormones than older leaves which are transitioning to the source status. Additionally, younger leaf tissues are expected to be etiologically plastic, making them amenable to developmental modifications. This enhanced delivery of hormones designed to stimulate regeneration maximizes the totipotent capability of these young leaves resulting in

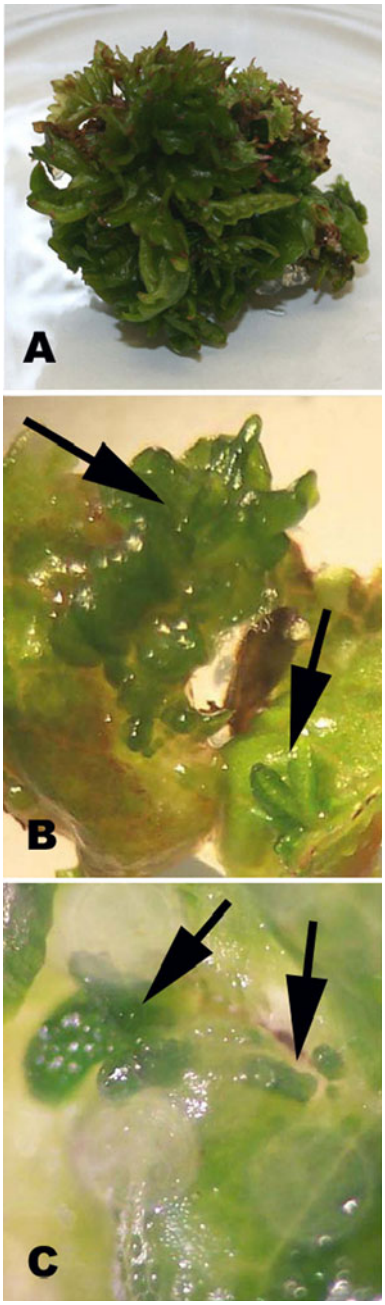

Fig. 3 Direct shoot organogenesis. **a** Prolific regeneration of explant in Trial 4, approximately 3.5 cm in diameter; **b** and **c** *Arrows* point to areas of regeneration in proximity to vascular tissue

successful shoot organogenesis. In addition to being strong sinks, the higher rate of regeneration of age 0 leaves may be related to their growth primarily by cell division (Fig. 1a, b). It is likely that the regenerative capability of age 0 leaves was the result of a combination of these two factors along with other physiological and/or biochemical processes unique to leaves in this stage of development. Secondary to leaf age and developmental stage, it was found that pre-conditioning treatments to the source plants prior to the excision of the explant material helped to ameliorate the stress of excision from the plant and plating on media. Reduced stress to the explant resulted in reduced browning and tissue death, and enhanced regeneration capability.

Data analysis was first performed with a full logistic regression model which included treatment, leaf age, and an interaction term of treatment x age as predictors of successful organogenesis. The logistic regression model incorporating both age 0 and age 1 leaves had one significant regressor, leaf age (Chi-square $p < 0.0001$). Treatment effect was not significant (Chi-square $p = 0.2269$) due to the low organogenesis rates associated with the age 1 leaves (Fig. 2). The interaction effect of treatment X leaf age was consequently non-significant as well (Chi-square $p = 0.6456$). A second, simplified logistic regression model involved only the treatment effect on organogenesis in age 0 leaves. The effects of treatment were marginally insignificant (Chi-square $p = 0.0606$) at $\alpha = 0.05$. Therefore, orthogonal contrasts were used to explore the effects of specific treatments relative to the control in the age 0-only model. The Dark-only and Soak-dark treatments both produced significantly higher odds of organogenesis than the control ($p = 0.0181$ and 0.0196, respectively). The Dark-soak treatment proved to be marginally insignificant ($p = 0.0606$). The Vacuum-dark treatment did not differ from the control ($p = 0.1950$).

In comparison to the control, all of the treatments in age 0 leaves resulted in increased regeneration (although not all increases were statistically significant) with the most significant feature of treatment being the dark period of 24 h prior to explant excision. It has been shown that photosynthesis decreases during prolonged dark period leading to a decrease in starch accumulation (Taiz and Zeiger 2006). Such phenomena may have helped the excised plant in relieving subsequent stress by minimizing the amount of chloroplast starch that is degraded to produce sucrose, thereby reducing the amount of stress-inducing sucrose in the explant. It is plausible that the added treatments of vacuum infiltration and complete submersion in liquid VRM during the soak caused enough plant stress to counteract the benefit of the dark period to a certain extent.

It has been established that the regeneration capability of *V. vinifera* is dependent on variety, and Pinot Noir and Pinot Meunier have proven to be two of the most difficult to elicit regeneration (Peros et al. 1998). Since Pixie is a descendent of these two varieties, the success of this protocol has the potential to contribute to a more stable process of regeneration for all standard grape varieties from the most recalcitrant to the most responsive. Further research will apply this protocol to standard varieties of *V. vinifera* with the objective of providing a genetically more stable method of regeneration for the purposes of propagation and transformation research.

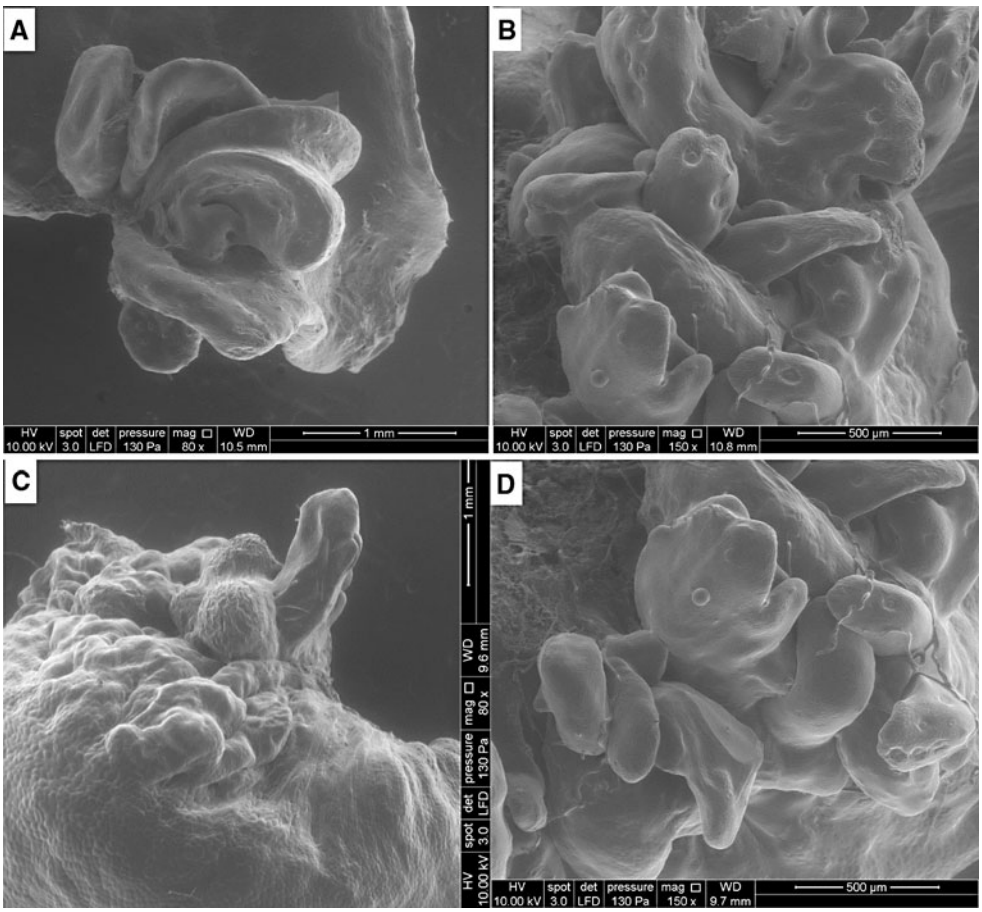

Fig. 4 Field emission scanning electron microscopy images. **a** Ruffle-shaped structure emerging from pleated plant tissues; **b**, **c** Shoot primordia emerging in multiples or singly; **d** Organogenic callus

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