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Sweet Cherry (*Prunus avium* L.) 'Giorgia', Adventitious Regeneration from Leaves of Microplants

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Summary

With the aim of exploiting the regenerative capability of sweet cherry (*Prunus avium* L.) 'Giorgia', experiments were carried out using suitable plant tissue cultures established in vitro. Relatively high percentages of adventitious shoot regeneration were consistently obtained from in vitro leaves, wounded by three cuts transversely to the midrib, excised from 3-week-old proliferating shoots. The regeneration capacity was influenced by the mineral salts and the plant growth regulator (PGR) composition of the regeneration medium: Woody Plant Medium (WPM) gave the best results and 22.7 µM of 1-Phenyl-3-(1,2,3-thiazol-5-yl)urea (thidiazuron, TDZ) induced most adventitious regeneration. The pre-culture of the donor shoots on MS medium with phloroglucinol (PG)-added improved the regeneration rate. Histological studies demonstrated the origin of the adventitious shoots from callus formed by epidermal and sub-epidermal cells as well as by the inner cells of the vascular bundles.

Key words. Fruit trees – recalcitrant species – organogenesis – tissue culture – thidiazuron – histology.

Introduction

Sweet cherry (*Prunus avium* L.) is one of the most popular of the temperate fruit crops, the production being concentrated in USA, Turkey and Italy. Up to the 1970 s, traditional scion varieties and vigorous rootstocks persisted in commerce (Bargioni 1996). Since then, new improved varieties with larger fruit and pathogen resistance characteristics have been bred, self-fertile varieties have been developed and dwarf rootstocks have been identified. However, the evaluation of a conventional breeding programme requires time-consuming observation of long-term breeding cycles and, given the high levels of heterozygosity of several fruit tree crops, a large segregating population of trees. Thus, the application of new technologies to the genetic improvement programme is important for a more rapid progress in the development of new fruit-bearing cultivars.

Regeneration of plants from somatic tissue or callus by organogenesis or somatic embryogenesis is a pre-requisite for any programme aiming to improve crop quality by genetic transformation. In fact, most of the biotechnological methodologies are dependent upon reliable methods of regenerating plants from cells or tissues (Horsch et al. 1985). Fruit trees are among the most recalcitrant species for in vitro culture and up to now regeneration protocols from mature somatic explants have been developed in only few fruit tree crops.

In sweet/wild cherry (*Prunus avium* L.), successful results have been obtained with roots (Gutierrez-Pesce et al. 1998) or leaves (Yang and Schmidt 1992; Hammatt and Grant 1998; Grant and Hammatt 2000; Piagnani et al. 2002; Tang et al. 2002; Bhagwat and Lane 2004) and from leaf protoplasts (Ochatt 1991). Few of these reports are relative to commercial sweet cherry fruit cultivar (Tang et al. 2002; Bhagwat and Lane 2004).

The purpose of this investigation has been to get new insights into the development of the experimental conditions for the adventitious regeneration from leaves of the important commercial cultivar 'Giorgia' of sweet cherry, as a necessary first step in a plan of a genetic transformation for this cultivar. A histological study of the regeneration process is also presented, in order to investigate whether the origin of the adventitious shoot primordia was from the newly formed callus or directly from the explant tissues.

Materials and Methods

Abbreviations

BA: N^6-benzyladenine; DKW: Driver and Kuniyuki medium (1984); FAA: formalinacetic acid-alcohol; IBA: indole-3-butyric acid; MS: Murashige and Skoog medium (1962); MM: multiplication medium; NAA: α-nphthaleneacetic acid; PG: phloroglucinol; PGR: plant growth regulator; LP: Quoirin and Lepoivre medium (1977); RM: regeneration medium; SEM: shoot elongation medium; TDZ: thidiazuron (1-Phenyl-3-(1,2,3-thiazol-5-yl)urea); WPM: woody plant medium (Lloyd and McCown 1980).

Maintenance of shoot cultures

Shoot cultures of sweet cherry (*Prunus avium* L.) 'Giorgia' were established and propagated in vitro. Micropropagation was carried on by sub-culturing shoots at 3-week intervals onto a shoot multiplication medium (MM). In order to find the best conditions for the multiplication and subsequently regeneration of this sweet cherry cultivar, several basal media were tested, based on the mineral salts and vitamins indicated by Murashige and Skoog (MS) (1962), or by Driver and Kuniyuki (DKW) (1984). All culture media were supplemented with 3 g L^{-1} sucrose and 0.6 g L^{-1} agar (Plant agar, Duchefa). The pH was adjusted to 5.7 with 1 M KOH before autoclaving for 20 min at 121 °C. Prior to autoclaving, shoot multiplication media were supplemented in turn with 1 mM PG (Sigma), 2.2 µM BA and 0.2 µM IBA. All cultures were maintained in a growth room at 25±2 °C, with a 16 h daily photoperiod of 120 µmol m^{-2} s^{-1} provided by Philips TLD/83 white fluorescent tubes, positioned 40 cm above the level of the cultures.

Regeneration from leaf explants

Young expanding leaves were excised from 3-week-old proliferating in vitro shoots. Each leaf was transversely cut (not completely) in the midrib three or four times and cultured with the abaxial surface in contact with the regeneration medium (RM) in 9 cm diameter Petri dishes (25 ml $dish^{-1}$). The basal media WPM and LP, both with the addition of 30 g l^{-1} sucrose, 6 g l^{-1} agar (Plant agar, Duchefa), pH 5.7, were evaluated. RM were supplemented after autoclaving with filter-sterilized TDZ or BA (from 2.5 to 68.1 µM) and 0.54 µM NAA. Tolerance tests to TDZ were performed using a wide range of cytokinin concentrations (from 0 to 68.1 µM). The effect of various culture media on the donor shoots (prior to leaf excision) was also investigated by taking leaves from shoots cultured on the following multiplication media: MM1 (MS basal media plus 2.2 µM BA and 0.2 µM IBA), MM2 (MM1 plus PG), MM3 (DKW basal medium plus 2.2 µM BA and 0.2 µM IBA), MM4 (MM3 plus PG). For each treatment, five leaves were cultured per Petri dish and six replicates were made. The experiments were repeated at least twice. The treatments were performed in a completely randomised design. During the induction period, all explants were kept for 30 days in the same conditions of light and temperature as indicated for the shoots.

Shoot initiation and development

Following 30 days incubation on the RM media, leaf explants were transferred to SEM, a MS basal medium without growth regulators, supplemented with 1 g l^{-1} activated charcoal. The following data were collected 30 days later: number of leaves regenerating shoots, number of sites of regeneration per leaf, location of regeneration sites per leaf. Developed shoots (5–10 mm height) were excised and micropropagated on MM1.

Statistical analysis

Data concerning the number of leaves regenerating shoots or not were analysed by contingency tables and χ^2-test (at a 95 % confidence interval). In line with the statistical theory, binomial data were also analysed by logistic regression (Mize et al. 1999) relating the logarithm of the successful outcomes (regenerated shoots) to the linear regression of TDZ combinations. Mineral media and cytokinin effect on regeneration percentage (number of proliferating shoots/total leaf explants) on TDZ media were subjected to one way analysis of variance, and differences between means were evaluated using the Tukey test ($P \leq 0.05$). Percentage mean values were subjected to arcsine angular transformation before analysis. All the experiments were repeated twice to confirm the results observed in the first experiment (by GraphPad Software) (Motulsky 1999).

Histological analysis

Five leaf explants were fixed in FAA (70 % ethanol:glacial acetic acid:formalin=18:1:1 v/v) every four days until the end of culture (30 days) on RM. Fixed explants were dehydrated through a 3-butanol series, embedded in Paraplast-XTRA paraffin (melting point 50–54 °C, Oxford Labware) and sectioned at 20 µm intervals with the automated microtome, Reichert-Jung 2050. Sections were stained with Eosin and Carazzi's haemalum (Mazzi 1977), purchased from Merck, mounted with Eukitt and observed under the Leitz Orthoplan microscope.

Results

Adventitious regeneration occurred only in RM with WPM salts and vitamins, LP-based media being non-promoting regeneration (data not shown). The effects of mineral salts and vitamins composition of the shoot multiplication media (preconditioning), as well the effect of phytohormone composition of the regeneration media on adventitious regeneration from leaves were considered.

1. The effect of the preconditioning of donor shoots on organogenesis induction.

Organogenesis from leaf explants was affected by different preconditioning treatments of donor shoots prior to leaf excision. Only at high TDZ concentrations (30 and 35 µM) was adventitious shoot regeneration observed from leaves taken from shoots subcultured on the MM1 medium (data not shown), whereas at all TDZ concentrations regeneration occurred from leaves taken from shoots subcultured on MM2 medium (Table 1). When shoots were subcultured on a medium with DKW salts, regeneration from leaves was observed only on the PG added medium (data not shown), and at a lower rate than with preconditioning on the MS medium.

2. The effect of growth regulators on organogenesis induction.

Since it is well known that BA and TDZ are the most effective cytokinins for promoting regeneration in recalcitrant species, we first tried BA and TDZ at four concentrations (2.5, 5.0, 10.0, and 22.7 µM), in combination with 0.54 µM NAA. Adventitious regeneration was induced on all media containing TDZ, while media containing BA did not promote organogenesis at any concentration tested so far (data not shown).

The chi-square test between the TDZ concentration employed in the RM and the event regeneration/no regeneration did not show a large value of chi-square, indicating that there was not a large discrepancy between the

Table 1. Effect of TDZ on regeneration of sweet cherry 'Giorgia' leaf explants placed on WPM with several TDZ doses added (NAA concentration was fixed at 0.54 μM). Microplants were taken from MM2 medium. Data are from a twice repeated experiment (n=20; SE was less than 10 % in all the values).

Medium	TDZ (μM)	% of leaves that gave rise to adventitious shoots	n° of regenerated shoots	average number of rege-nerated shoots per explant
RM1	2.5	5	2	0.10
RM2	5.0	15	7	0.35
RM3	10.0	5	1	0.05
RM4	22.7	25	13	0.65
RM5	30.0	10	9	0.45
RM6	35.0	25	15	0.75
RM7	45.4	5	2	0.10
RM8	68.1	10	2	0.10

treatments. Contingent table showed 7.3 χ^2 and a P-value (0.06) indicating that there is a scarce association between the regeneration phenomenon and the TDZ concentration. However, the logistic regression performed on the logarithm of the successful outcomes (regenerated shoots) to linear regression of TDZ combinations, showed a goodness of fit to the linear regression with r^2=0.735. Thus 74 % of the total variance of the observed regenerated explants was explained by the linear regression model demonstrating a linear relationship between regeneration and TDZ concentrations (Fig. 1).

After tolerance tests carried out in order to fix the threshold of toxicity and the range of morphogenic activity of TDZ, it was concluded that toxicity on leaf explants was very low, since the limit of tissue browning was over 68.1 μM TDZ. The range over which TDZ performs a morphogenic activity is very wide (from 2.2 to 68.1 μM). The highest regeneration rate (25 %) was observed on media RM4 and RM6, which contained WPM mineral salts and vitamins plus 22.7 μM TDZ / 0.54 μM NAA and 35 μM TDZ / 0.54 μM NAA, respectively. One of the most efficient TDZ concentration was 35 μM concerning the percentage of regeneration and it was also the most efficient concentration in terms of the number of regenerated shoots (Table 1).

At high TDZ concentration (more than 30 μM), however, regenerated shoots showed hyperhydricity. For this reason the subsequent experiments were conducted employing only the range of 2.5–22.7 μM concentration. Results for the analysis of the variance of the regeneration percentage showed statistical differences in mean values relative to the 22.7 μM TDZ concentration as shown in Fig. 2.

In order to induce adventitious regeneration, the most efficient TDZ concentration is, therefore, 22.7 μM. In this conditions, more than one and up to six adventitious shoots were observed to develop from the same regeneration site which usually was proximal to the petiole. The

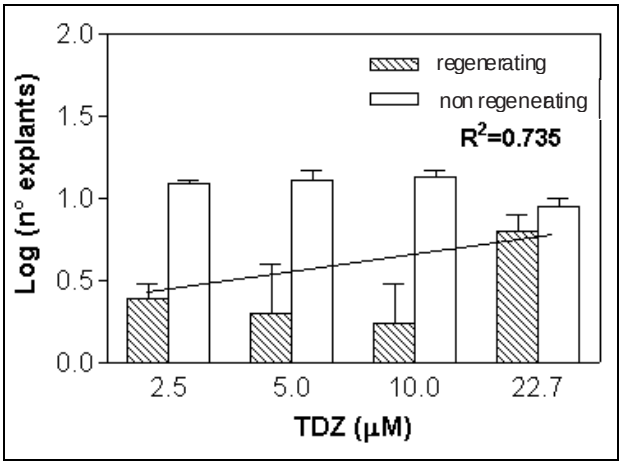


Fig. 1. Mean number of regenerating/non regenerating explants ('Giorgia' leaves) ± SE (n=15) transformed in the logarithmic scale versus TDZ combinations. Logistic regression was performed relating the logarithm of the successful outcomes (regenerated shoots) versus the TDZ combinations (Graph Pad Software-Prism 3, 2002).

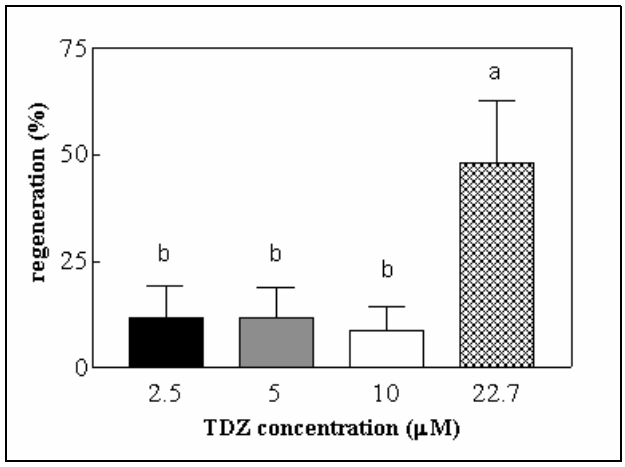


Fig. 2. Effect of TDZ concentration on regeneration from leaf explants of 'Giorgia' sweet cherry (RM media contained WPM mineral salts and were added with different TDZ doses and 0.54 μM NAA). Percentages were subjected to arcsin tranformation before ANOVA analysis. Values are means ± SE (n=30). Different letters correspond to significant different means (P≤0.05).

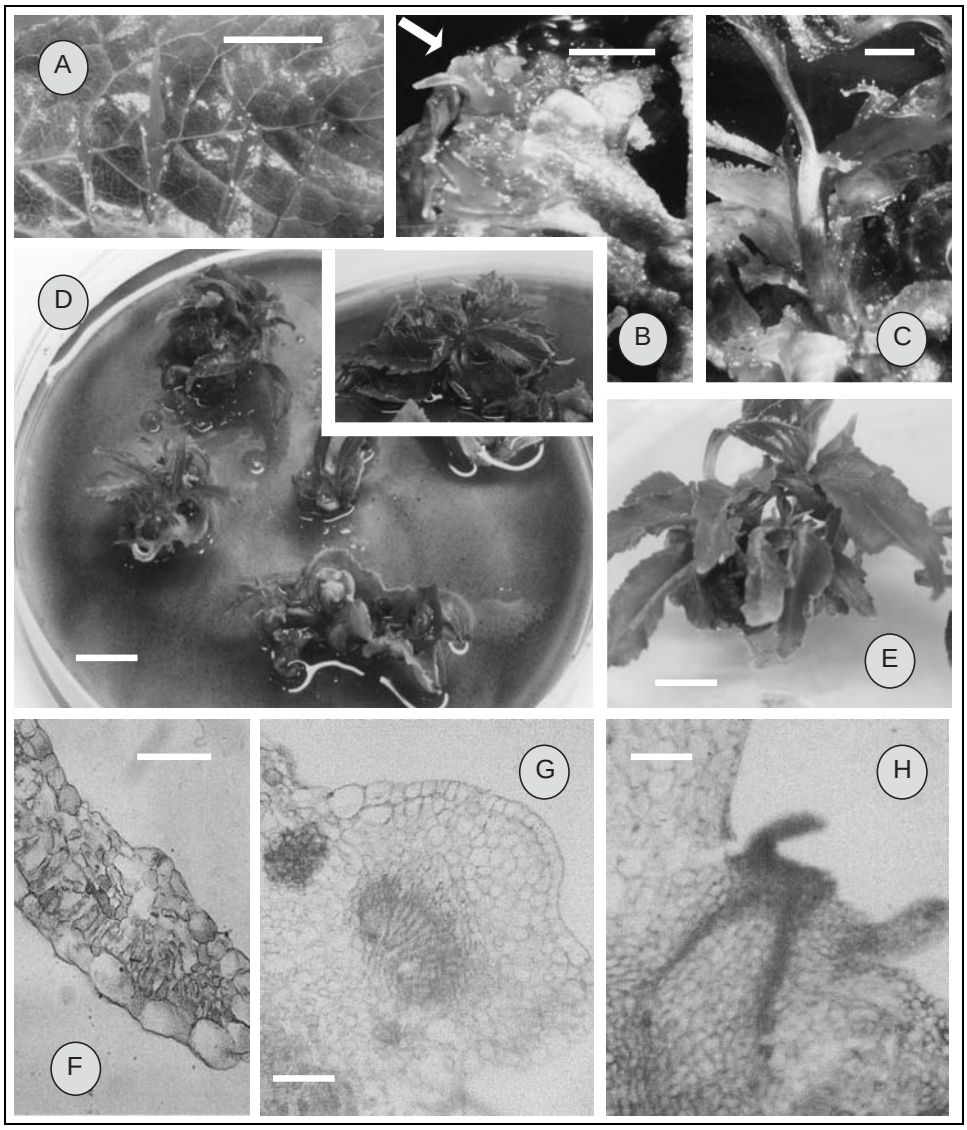


Fig. 3. (A-E) Adventitious shoot regeneration from leaves of *Prunus avium* L. 'Giorgia'. (A) Leaf transversely cut in the midrib at the beginning of the culture on regeneration media (bar: 0.4 cm). (B) Adventitious bud (arrow) developing from green callus tissue produced on the leaf explant after 20 days of culture on RM4 (bar: 0.4 cm). (C) Regenerated adventitious shoot 10 days after transferring leaf explant to SEM (bar: 0.3 cm). (D) Leaf explants 20 days after transfer to SEM (bar: 0.3 cm), showing high regeneration frequency (a particular of regenerated shoot in the square) (bar: 1.25 cm). (E) Adventitious regenerated shoot, after excision from the original explant, ready to be transferred to MM1 (bar: 1 cm). (F-H) Histological time course of the adventitious bud formation from leaf explant on RM4. (F) Leaf section, at the beginning of the culture (t_4) on RM4 (bar: 50 μm). (G) Vegetative primordium emerging from a superficial proliferated area (bar: 100 μm). (H) Vegetative bud showing distinct vascular connection (bar: 100 μm).

time course of the regenerative process is presented in Fig. 3A–E.

Histological analysis

The analysis of the histological sections of the regenerating leaves allowed us to follow the time course of the regenerative process, from the setting up of the explants to the development of the regenerated shoots.

In leaf explants cultivated on a regeneration medium (RM4) for a few days (t_4), a light proliferation of the bundle-sheath cells and of the tissue surrounding the vascular bundle could be observed (Fig. 3F). During culture time, the morphology of the leaf tissue changed progressively. Cellular proliferation from epidermal and sub-epidermal cells as well from the cells near the vascular bundles became more evident. In some cases non-organized cellular proliferation occurred with callus production, while in other cases xylogenesis and vegetative primordia were observed, arising from the callus (Fig. 3G). These structures subsequently developed into vegetative buds (Fig. 3H).

Discussion

Few works on sweet/wild cherry regeneration are reported compared to regeneration studies of other fruit tree species (Ochatt 1991; Yang and Schmidt 1992; Hammatt and Grant 1998; Grant and Hammatt 2000; Gutierrez-Pesce et al. 1998; Piagnani et al. 2002) and particularly on sweet cherry cultivars (Tang et al. 2002; Bhagwat and Lane 2004).

Our work on sweet cherry 'Giorgia' regeneration was aimed at exploiting the capability for regeneration of this important commercial cultivar by establishing the experimental conditions for a good and efficient regeneration, in order to plan its genetic transformation by *Agrobacterium tumefaciens*. 'Giorgia' is an Italian cultivar, introduced in 1985, selected by G. Bargioni at the Istituto Sperimentale Frutticoltura of Verona, Italy. The cultivar has interesting characteristics as the dimension of the fruit, the moderate susceptibility to cracking, the yield precocity and the high productivity.

Our results regarding sweet cherry 'Giorgia' regeneration from in vitro leaves confirm the reports on the regen-

erative capability found in other sweet/wild cherry genotypes. In our system too, WPM basal medium worked well in inducing regeneration (HAMMATT and GRANT 1998; GRANT and HAMMATT 2000; BHAGWAT and LANE 2004) and better than LP mineral salts and vitamins, as well as reported by TANG et al. (2002). The result of the preconditioning/regeneration interaction revealed that the MS-based medium was more effective than DKW for preconditioning donor shoots on organogenesis induction. Moreover, the addition of PG to multiplication medium in order to improve the shoot proliferation, seems to influence even the successive experiments of regeneration. PG is a phenolic compound widely studied since the 1970 s. We used PG to improve shoot proliferation, as reported in wild cherry by HAMMATT and GRANT (1993). It is hypothesised that PG is a regulator of the redox state of the cell, keeping the cell in a reduced state, a favourable condition for cellular division and organ differentiation (STONIER 1972). Moreover, PG, being a scavenger, protects free auxin from oxidative degradation (LEE 1980).

We did not find adventitious regeneration on BA supplemented media, as some authors report (YANG and SCHMIDT 1992; HAMMATT and GRANT 1998), and in great contrast with TANG et al. (2002), who find BA more effective than TDZ in regeneration. However, our results confirm the reports of HAMMATT and GRANT (1998) and BHAGWAT and LANE (2004), who found the occurrence of regeneration more frequently on medium with TDZ rather than BA. On the other hand, even in other genotypes, as 'Hedelfinger', if BA replaced TDZ organogenesis did not occur (PIAGNANI et al. 2002). It is now well documented that, in woody plants, cytokinins such as TDZ work better than adenine-based cytokinins (such as BA), even if the mechanism of TDZ action is not fully understood (HUETTEMAN and PREECE 1993).

Considering that the contingent table of the four different TDZ concentrations demonstrated that they were not associated strongly enough with the regeneration events to be statistically relevant, it was decided to perform a logistic regression. The linear model of the logarithm of the number of regenerating explants *versus* TDZ concentrations allowed us to predict that the regeneration event occurred with a chance of 73 % as shown by the regression line trend. Even if the number of regenerating events per explant is definitely low, the Anova test regarding the overall regeneration percentage showed a significant difference between TDZ treatments and, considering the number of regenerated shoots arising from the same regeneration site, in some cases up to six shoots regenerated from one cultured leaf.

Several factors are important when studying the regenerative capability of a plant species, especially the recalcitrant ones. Type and concentration of PGR, size of explant, leaf surface orientation are important parameters which need to be considered. We used a whole-leaf explant wounded by cutting through the midrib, a kind of explant which is demonstrated to be the most regenerating one both in cherry (HAMMATT and GRANT 1998; GRANT and HAMMATT 2000; BHAGWAT and LANE 2004) than in other fruit species, as apple (FASOLO et al. 1989), quince (DOLCET-SANJUAN et al. 1991), almond (MIGUEL et al. 1996), wild pear (CABONI et al. 1999), and apricot (PEREZ-TORNERO et al. 2000). As the leaf surface orientation is concerned, we used the abaxial surface in contact with the RM, as reported in other regeneration studies on cherry (HAMMATT and GRANT 1998; GRANT and HAMMATT 2000). In order to improve the regeneration efficiency, we are planning to test even the opposite situation, since in a thorough study on adventitiuos regeneration of two cherry cultivar, both situations have been tested, and regeneration was promoted by incubating explants with the adaxial surfaces in contact with the medium (BHAGWAT and LANE 2004).

The histological analysis of the regeneration process is of fundamental importance with regard to the identification of the origin of the adventitious shoot primordia and in following the developmental stages of organogenesis. Previous studies on adventitious regeneration in sweet/wild cherry have not reported a histological analysis. Our histological studies on 'Giorgia' sweet cherry regenerating leaves show that adventitious shoots develop from the morphogenic callus. As reported in other woody plant systems, organogenesis from callus is a "guarantee" of the avoidance of chimeral transgenic plant production (RUGINI et al. 1991), even if somaclonal variation can occur (EVANS and SHARP 1986). The progeny of the regenerated plants we obtained from sweet cherry leaves did not show phenotypic variation, although no genetic analysis has been performed so far.

In conclusion, the regenerative potential revealed by the sweet cherry fruit cultivar 'Giorgia', following in vitro manipulation applied to leaf explants, promises to establish one of the basic requirements for its *Agrobacterium*-mediated genetic transformation.

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