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Shoot Regeneration, *in Vitro* Performances of Regenerated Shoots and Transient Expression in Morphogenic Explants in *Prunus avium* cultivar 'Burlat C1'

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Summary

Biotechnology may represent alternative ways to genetic improvement of sweet cherry exploiting somaclonal variation and genetic transformation. In this paper a method to induce efficient shoot regeneration from somatic explants of 'Burlat C1' is reported: 52.0 % caulogenesis was obtained from basal callus formed after culturing shoot apical portions in the dark. Considering the significant onset of adventitious shoots showing variegated leaves their in vitro performances have been evaluated as compared to green (normal) shoots. The influence of cefotaxime, cefotaxime plus vancomycin and timentin was assessed both for decontamination and regeneration efficiency prior transforming morphogenic masses with *A. tumefaciens* (C58C1 pDN3514), carrying the rolABC genes from *A. rhizogenes*, p35GUS-intron visible marker and nptII antibiotic resistance gene. GUS test indicated the presence of transient expression in shoot primordia nevertheless it was not possible to recover transgenic shoots probably due to the formation of chimeric meristems.

Key words. *A. tumefaciens* – acetosyringone – cefotaxime – leaf variegation – timentin – vancomycin

Introduction

Intensive fruit tree growing requires cherry orchards to be manageable from the ground with a medium-high planting density to reduce the cost of harvesting and pruning. Biotechnology and in particular the use of genetic transformation (Druart et al. 1998; Piagnani and Scotti 2006; Song and Sink 2006, 2007) and somaclonal variation (Piagnani et al. 2002, 2008) is particularly important for those species such as cherry, for which the introduction of the compact and spur habitus, according to the traditional breeding programs, is made complex by the fact that the segregation of this polygenic traits has proved largely haphazard and weakly associated with its parental frequency (Sansavini and Lugli 1996). In Europe, among the earliest cultivar, 'Burlat' is grown mostly for export to foreign markets, but being highly perishable it is difficult to maintain suitable quality conditions during transportation (Remon et al. 2006). Genetic transformation offers a promising approach to make the fruits more resistant to damage during harvesting, processing and shipping (Callahan and Scorza 2007). The nature of any modification induced by both, somaclonal variation and transformation, can be verified only if an efficient regeneration procedure is available while *in vitro* multiplication of mutated/transformed shoots allows to speed up testing of variant/transgenic plants. Also for sweet cherry the genotype response represents a bottle neck for a successful morphogenesis and *Agrobacterium* mediated transformation (Tang et al. 2002; Piagnani et al. 2002; Piagnani and Scotti 2006); considering this, the critical steps of in vitro culture such as culture initiation and adventitious meristems induction (caulogenesis) as related to subsequent transformation protocol should be set up on cultivar base. 'Lapins' has been the most investigated among sweet cherry cultivars and considering the satisfying results obtained it can be defined as respondent to the in vitro culture. Differently from cv. 'Lapins' (Bhagwat and Lane 2004; Piagnani and Scotti 2006; Feeney et al. 2007) cvs. 'Hedelfinger' and 'Burlat' in particular showed recalcitrant to shoot production from adventitious meristems (Piagnani et al. 2002). In the literature the only paper on 'Burlat' shoot regeneration, starting by leaf explants (Tang et al. 2002) showed regeneration efficiency ranging from zero to such low levels to be inadequate for supporting genetic transformation or somaclonal variation. The mutant C1 of cv. 'Burlat' is considered more than its parental as more productive, for the speed of fruiting and because less vigorous; nevertheless the decrease in vigour is still not sufficient to overcome the slow entry into production. In this paper a method to induce efficient shoot regeneration from somatic explants of 'Burlat C1' is reported and considering the significant onset of adventitious shoots showing variegated leaves the in vitro performances of regenerated shoots have also been evaluated. In preliminary assays it was found that by using a previous protocol set up for cherry (Piagnani and Scotti 2006) although a high percentage of decontaminated explants could be obtained none was able to produce even a small amount of callus and turned brown quite soon (not published). This behaviour was ascribed to the decontamination procedures which caused explant stress and, as consequence of this our previous protocol was adjusted to obtain viable explants expressing the inserted genes.

Materials and Methods

Plants and culture conditions

Axenic shoot cultures from adult plants of the cherry cultivar 'Burlat C1' ('BC1') were grown on PM medium containing 5.0 μM BAP (6-Benzylaminopurine) and composed of MS (Murashige and Skoog 1962) minerals, Nitsch and Nitsch (1969) vitamins, 20 % (w/v) sucrose, 4.5 % (w/v) Bac agar (DID, Milan), pH was adjusted to 5.6 with KOH 1 N. Shoots were subcultured every twenty days in fresh medium, into 500 cm^3 transparent Linfa-boxes (Micropoli, Italy) each containing twelve shoots. Cultures were routinely grown at 22±1 °C, under cool white fluorescent tubes, irradiance of about 30 μmol s^{-1} m^{-2} and 16 h photoperiod.

Shoot regeneration

Shoots of about one cm in length were used and 5–6 mm apical portion of each shoot, including folded leaves was cut and cultured in 9 cm Ø Petri dishes containing 20 ml LP1 medium added with 5.0 μM BAP and 1 μM NAA (1-naphthalene acetic acid) according to Gentile et al. (2002), solidified with 4.5 % (w/v) Bac agar. After twenty days of incubation in the dark at 25 °C, both light brown spongy callus formed at the base of each shoot and the 3–4 mm upper stem portion deprived of axillary shoots/buds and leaves were separately subcultured on LP2 medium containing only BAP, in the light (Piagnani and Scotti 2006). The effect of the two explants was compared considering six different experiments of morphogenesis each performed on minimum 30 explants. Moreover, three different type of agar: Bac, Vitro and Industrial (Conda laboratories) at 4.5 % (w/v) were compared using the explant type which gave better result.

Data collected after 10, 20, 30, 40, 50 and 60 days were expressed as percentage of explants differentiating shoots.

In vitro *growth of regenerated shoots*

Proliferation. In this test, morphogenic calli grown on LP2 medium for 40 days were used. Twenty (b1 to b20) regenerated shoots about 0.3 cm in length were randomly selected and cut. Each shoot was then transferred to Coulters® containing 10 ml of LP2 and 2.5 μM BAP to stimulate shoot proliferation and elongation. Each b-shoot produced a shoot cluster on LP2 and each cluster was separately transferred to fresh PM with 2.5 μM BAP to allow shoots to adapt to routine culture conditions. At this time each regenerated shoot (bn) gave rise to one regenerated clone (Bn) (Fig. 1). To stimulate the formation of new sprouts for performing the proliferation trial, in some cases shoots were cut transversely in two or more nodal segments. At the end of the third subculture, 10 shoots of 0.5–1.0 cm in length from both B-clones and 'BC1' were selected and shoot total number and shoot length were recorded to compare B-clones and 'BC1' proliferation ability.

After the second subculture shoots showing variegated leaves arose. In order to quantify this phenomenon in six different regeneration experiments, regenerated shoots were subsequently monitored for the appearance of leaf variegation at the end of each of four subcultures. Shoots were cultured into Linfa-boxes, 10 shoots/each, so the percentages of shoots showing leaf variegation could be calculated on a basis of 10 shoots (class 10–100). At the end of the fourth subculture it was possible to calculate the percentage frequency with which each class was expressed.

Rooting. The purpose of this assay was to obtain in vitro rooting of regenerated B-clones (B1 to B20) and to compare rooting ability with that of 'BC1'. Once a suitable number of shoots for all B-clones was obtained, shoot rooting was achieved by basal dipping in 50 % ethanol solution of 3 mM filter (Agrodisc® Siringe Filters, 0.2 μm Ø) sterilised IBA (Indole-3-butyric acid) followed by the culture on growth regulator free half strength PM medium. For each Bn and 'BC1' a total of four Linfa-boxes containing 75 ml solidified medium with ten shoots each were used in two different experiments. Thirty days after the treatment the following parameters were recorded: number of rooted shoots, root number and length, and callus production. Rooting efficiency of leaf variegation phenotype and standard phenotype was also compared.

Transformation

Influence of antibiotic on regeneration. Antibiotics are used to eliminate *Agrobacterium* after co-culture. Cefotaxime or carbenicillin have been the most commonly used to eliminate *Agrobacterium* from plant material. The purpose of this experiment was to test the influence of cefotaxime, cefotaxime plus vancomycin and timentin on regeneration and to evaluate the best antibiotic combinations to use in the subsequent transformation experiments. Shoots of 1 cm in length were used and about 5 mm apical portion including folded leaves were treated as reported in the preceding pages. 8–11 explants (basal callus) were placed in each 9 cm Ø Petri dish. The following antibiotics were added to LP1 and LP2 medium: 200 mg l^{-1} cefotaxime, 200 mg l^{-1} cefotaxime plus 200 mg l^{-1} vancomycin, 250 mg l^{-1} timentin (Nauerby et al. 1997; Song and Sink 2007) control (no antibiotics). Data from three experiments were pooled after 20 days from light exposure.

Agrobacterium mediated transformation protocol. *A. tumefaciens* (C58C1 pDN3514), carrying the p35SGUSIntron binary vector was used to infect plant material. Shoots of less than 1 cm in length were deprived of the unfolded leaves and lateral buds and either fully immersed or basal cut dipped in a suspension of liquid LP1 and 2.5×10^8 bacterium cells ml^{-1} (OD_{520} = 0.5) and incubated at 25 °C for 72 h. In preliminary trials, performed to determine the minimal concentration of antibiotic necessary to inhibit our *Agrobacterium* strain, we found that 150 mg l^{-1} cefotaxime inhibited microbial growth. Shoots were decontaminated by 30 min vacuum immersion in 2 mg ml^{-1} lysozime followed by orbital shaking in liquid LP1 plus 200 mg l^{-1} cefotaxime for 72 h. At the end shoots were transferred to a dark room on solified LP1 containing 200 mg l^{-1} cefotaxime. The addition of the selective agent (50 mg l^{-1} kanamicine) was delayed for two weeks when the new callus formed at the shoot base was separately cultured under light in 6 cm Ø Petri dishes containing agarised LP2 added with either 200 mg l^{-1} cefotax-

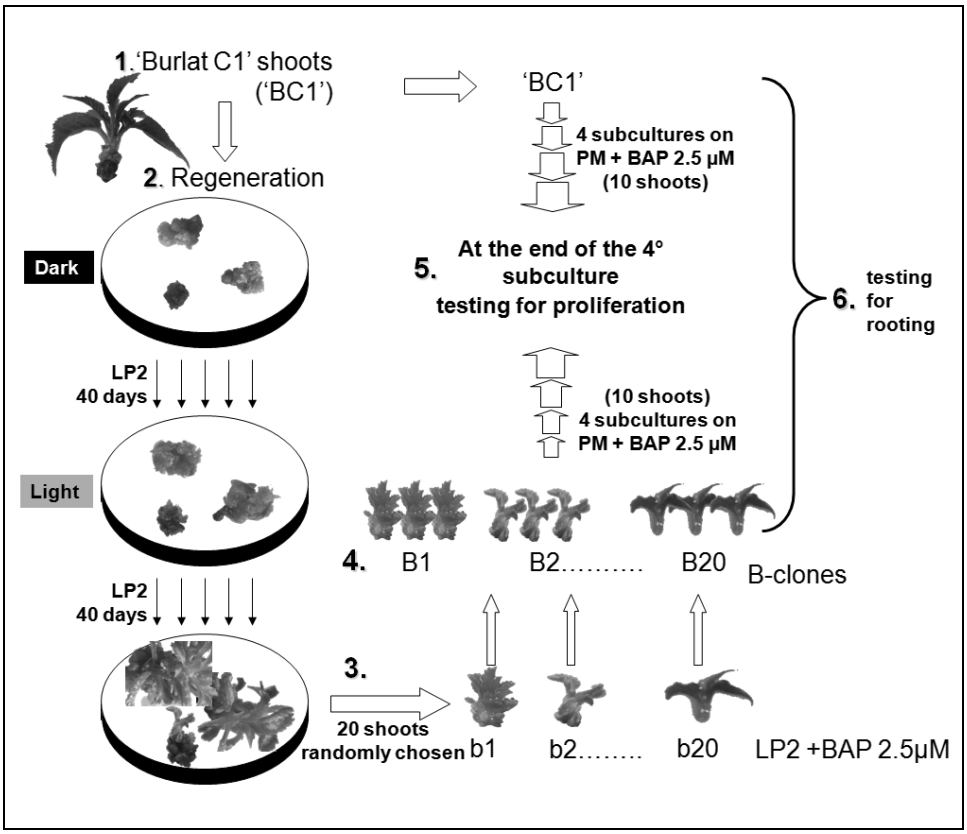


Fig. 1. Flow chart showing the experimental design. (1.) 'Burlat C1' ('BC1') *in vitro* cultured shoots. (2.) Apical portion of each shoot was cut and cultured on LP1 medium added with 5.0 µM BAP and 1 µM NAA, in the dark. After twenty days both spongy callus formed at the base of each shoot and the 3–4 mm upper stem were separately subcultured on LP2 medium containing only BAP, in the light. (3.) 20 shoots (b1–b20) about 0.3 cm in length, were randomly selected from regeneration clusters and transferred on LP2 and 2.5 µM BAP. (4.) b1–b20 started to proliferate giving origin to B1–B20 clones each cultivated separately on PM + BAP 2.5 µM for 4 subcultures. (5.) At the end of the 4th subculture shoots were tested for proliferation. (6.) Rooting ability of B-clones was also investigated.

ime alone or cefotaxime plus 50 mg l^{-1} kanamicine. This protocol used to transform other sweet cherry cultivars (Piagnani and Scotti 2006) was unsuccessful for 'Burlat C1' and considering this the following changes were made:

a) Introduction of *Agrobacterium* 'induction phase' including 500 mM acetosyringone, 20 mM sodium citrate and 2% sucrose, for *vir* genes overexpression (Gelvin 2006; Song and Sink 2006);
b) *Agrobacterium* concentration was lowered from 0.5 to 0.1 OD_{520};
c) Morphogenic calli only were co-cultivated with *A. tumefaciens*;
d) Co-cultivation was made on solid medium (instead of wet sterile paper);
e) Elimination of shoot rinsing in lysozime solution;
f) Decontamination made by washing the samples twice with 200 mg l^{-1} cefotaxime solution for 5 min and then trice with sterile water for 3 min each.

Six Petri dishes (9 cm Ø) each containing 9 co-cultivated explants were used.

GUS (β-glucoronidase) activity of infected explants was assayed histochemically (Jefferson 1987) after two weeks from the co-culture. The analysis was performed on new callus formed at the shoot base. Potassium ferricyanide 0.5 mM and potassium ferrocyanide 0.1 M were used as oxidation catalyst in order to stimulate colour production; differently from the original protocol 3.04 mM instead of 2.52 mM X-Gluc were used. Stained tissues were transferred to 70 % ethanol and incubated at room temperature to remove residual chlorophyll and improve the contrast of indigo dye. Data were recorded as presence or absence of blue spots (irrespective of size and number) in 12 samples randomly chosen in three separate groups and data were expressed as percentage. Photos were taken under a stereo microscope (Wild makroskop M420) connected to a digital camera (Canon Powershot S30).

Statistics

Data were analysed by one-way ANOVA and the differences contrasted using the Tukey's test. Statistical analysis was performed at 5 % level using SPSS 11.0 package for Windows by SPSS inc. Percentages were first subjected to Kolmogorov-Smirnov non parametric test of normality.

Results

Regeneration

Statistical data analysis revealed that the type of explant significantly affected regeneration rate in fact the percentage of calli undergoing morphogenesis, 52.0 %, was about twice as much as the stem, 29.2 % (Table 1). Contrariwise agar type did not affect shoot regeneration as the mean percentages shown did not differ significantly. In Fig. 2 the time course of shoot regeneration percentages and regression curves as calculated for each agar type is reported; independently on the agar, the maximum shoot regeneration can be achieved between 40 and 50 days from the beginning of the experiment.

In vitro *growing of regenerated shoots*

Proliferation. Proliferation coefficients of the twenty randomly selected shoots and 'BC1' are shown in Table 2. The coefficients calculated are covering a relatively wide

Table 1. Effect of explant and agar type on shoot regeneration percentage. Data were pooled 50 days after plating from six different experiments (n=6).

Explant type		Agar type		
Callus	Stem	Bac	Industrial	Vitro
52.0 b[+]	29.2 a	55.0 a	48.2 a	44.4 a

[+] Means with the same letter are not different according to the Tukey's test at 0.05 level

range, from 1.0 to 8.2, moreover five regenerated shoots on twenty, 25 % of total, showed significantly higher proliferation than the control. Shoot mean length did not differ significantly between Bn and the control and the values ranged from 0.6 and 1.3 cm (data not shown). Between the second and third subculture variegated leaf shoots started to occur in B-clones; this alteration was manifested in the form of depigmented (white) areas involving leaf surface to different levels of extension (Fig. 3). Fig. 4 shows the distribution percentage of regenerated shoots in the different classes of leaf variegation: classes 40 % and 80 % of leaf variegation occurrence are the most represented as occurring in 16.7 % of cases, unlike the less frequent are the classes 50 % and 100 %. No difference in term of shoot proliferation was detected between variegated and green shoots (data not shown).

Rooting. Rooting ability of B-clones (Bn) did not differ significantly from the 'BC1' (Table 3) and the variegated shoots did not significantly change root ability in comparison to the standard ones, in fact the percentages of rooting recorded for each type were 65.0 and 62.4 respectively. As regarding root mean number only one clone, B 9, scored a significant different value (7.6) in respect to both the 'BC1' (1.7) and clones B11, B18 and B19 (Fig. 5). B-clones differed from the 'BC1' in term of root quality as rooting was characterized by a significant positive correlation between rooting percentage and callus presence at the shoot base, contrariwise 'BC1' showed a significant but negative correlation (Table 3). *Ex vitro* plantlets showed a complete recovery from leaf variegation one-two months after transferring to green house.

Agrobacterium mediated transformation

Table 4 shows the effect of different antibiotics or antibiotic combinations on the regeneration process. The percentage of explants forming callus ranged between 43 % and 46 % when 250 mg l^{-1} timentin or 200 mg l^{-1} cefotaxime and vancomycin were used, in contrast the percentage raised to 68 % and 78 % when no antibiotics or only 200 mg l^{-1} cefotaxime were added to the culture medium. According to this, only cefotaxime was used in transformation experiments as decontaminant agent. In these conditions only few samples were fully decontaminated and still able to grow. In the different transformation experiments performed, the randomly assayed samples positive to GUS test ranged from 50 to 75 %, with the involvement of explant areas with different sizes (Fig. 6). After two to three months from infection differentiation process ceased showing whitening effects on morphogenic masses due to kanamycin action indicating that they were not fully transformed.

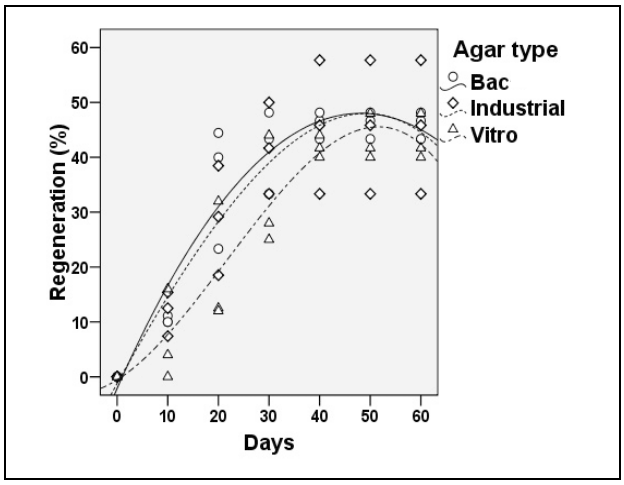


Fig. 2. Time course of shoot regeneration percentages and cubic curve interpolation as calculated for each agar type (Bac: R^2=0.9; Industrial: R^2=0.8; Vitro: R^2=0.9).

Discussion

This research was conducted with the aim of developing an appropriate protocol for in vitro morphogenesis and transient expression of cv. 'Burlat C1', which had shown recalcitrant to *in vitro* culture.

Factors which demonstrated to be crucial for morphogenesis in previous researches on recalcitrant cultures have been here considered. In morphogenesis trials it was confirmed that the level of morphogenic response is

Table 2. Proliferations coefficients as calculated for twenty B-clones and for the wild type, at the end of the forth subculture on PM with 2.5 μM BA (Inspected explants n=10).

B-clone	18	20	7	8	13	19	9	11	17	'BC1'	6	10	14	16	12*	5	1	2	3	15	4
Shoot proliferation coefficient	1.0	1.0	1.2	1.2	1.5	1.7	2.0	2.0	2.0	2.5	2.7	3.2	3.2	3.2	4.0	4.7	5.5	5.5	5.7	5.7	8.2
Significance letter	b[+]	b	b	b	b	b	b	b	b	b	ab	ab	ab	ab	ab	ab	a	a	a	a	a

* Data collected from three shoots because the other 7 were contaminated.
[+] Means sharing the same letter are not statistically different according to the Tukey's test at 0.05 level.

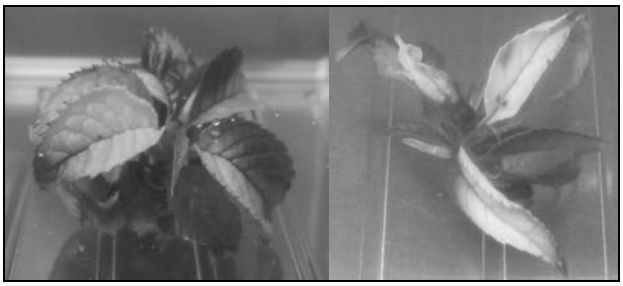


Fig. 3. Different patterns of leaf variegation in B-clones. Leaf variegation began to appear up to the end of the second subculture.

Table 3. Rooting percentages calculated for both standard (green) and variegated leaf shoots belonging to B-clones and to the wild type. Pearson's coefficient (r) showing a linear relationship between the two variables callus presence and rooting percentage.

Rooting (%)			Pearson's correlation coefficient (callus-rooting)	
B-clones standard	B-clones variegated	'BC1'	B-clones	'BC1'
62.4 a	65.0 a	67.5 a	0.356**	−0.543**

[+] Means with the same letter are not statistically different: Tukey's test at 0.05 level (n = 4). (**) at the 0.01** level (2-tailed).

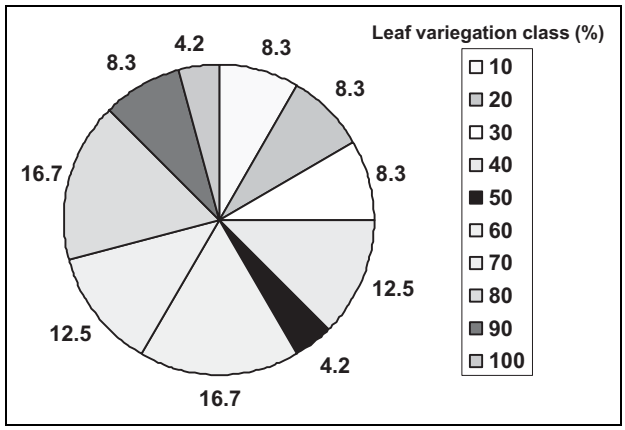


Fig. 4. Frequency of leaf variegation occurrence in B-clones. B-clones coming from different regeneration experiments were cultured in Linfa-boxes, 10 shoots/box, so the percentages (classes) of variegation ranged between 10 and 100. At the end of the fourth subculture the percentage frequency with which each class was expressed was calculated. 25 B-clones were considered, for each B-clone 1 to 5 Linfa-boxes were scored.

depending on explant type. In particular the best result was scored from callus and this is in disagreement with what happened previously on other sweet cherry cultivars (Piagnani and Scotti 2006). Unlike what obtained with peach, a recalcitrant species, in this case agar type did not affect morphogenesis (Piagnani et al. 2004). Proliferation of regenerated shoots highlighted a wide range of axillary bud disclosure potential and this aspect, related to apical dominance, needs to be better investigated as shoots retaining the highest proliferation coefficients could be somaclonal variants candidate for reduced apical dominance. Somaclone HS from cultivar 'Hedelfinger' was in fact isolated also according to its *in vitro* response to cytokinin (Piagnani et al. 2002). B-clones showed different rooting physiology compared to 'BC1' nevertheless this did not interfere with rooting efficiency. In all regeneration experiments, shoots with variegated leaves have been produced with different frequencies compared to the standard phenotype. It is the first time that a similar phenomenon is described for an *in vitro* grown temperate tree fruit in general, and in particular for cherry. Crossing three sweet cherry cultivars, Kerr (1963) found a partially albino phenotype in the offspring produced by and this probably was an "extreme" variegation of leaf lamina. Contrariwise, for subtropical fruit species the appearance of somaclones showing leaf variegation has already been described. In our case it seems likely that the emergence of variegated leaves is the expression of an epigenetic phenomenon as it occurs in a precise timing of the culture and then disappears fairly rapidly during *ex vitro* hardening. Epigenetic aspects of somaclonal variation are related to gene activation/

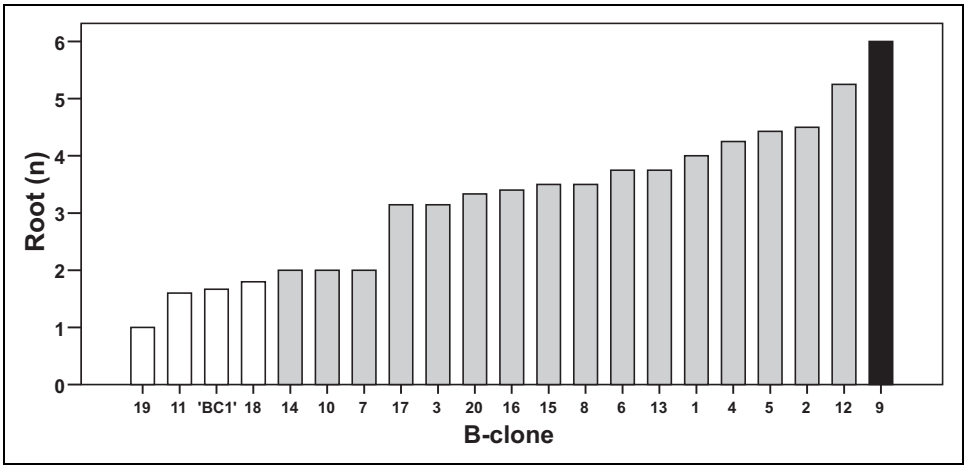


Fig. 5. Root mean number as scored for each B-clone and wild type. Black and white bars are statistically different according to the Tukey's test at 0.05 level, n=40.

Table 4. Effect of antibiotic type and combination on regeneration percentage. Data from three experiments were pooled.

Antibiotics	Regeneration (%)	Inspected explants (n)
Control	68 a	62
Cefotaxime	78 a	78
Cefotaxime + vancomycin	46 b	70
Timentin	43 b	73

[+] Means with the same letter are not different according to the Tukey's test at 0.05 level.

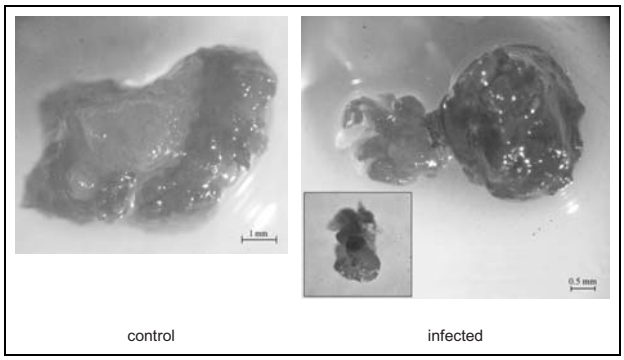


Fig. 6. Gus activity in morphogenic masses originated from basal callus of 'BC1' shoots after two weeks from co-culture with *A. tumefaciens* (C58C1 pDN3514), carrying the rolABC genes from *A. rhizogenes*. Data were recorded as presence or absence of blue spots (irrespective of size and number). Photos were taken under a stereo microscope wild makroskop M420 connected to a Digital Camera Canon Powershot S30. Bar =1 mm (control) and 0.5 mm (infected).

silencing mechanisms which do not involve any change in the base sequence of DNA or chromosomal aberration and, according to this, they are potentially unstable or reversible. Researches ongoing highlight the role of histone modifications, DNA methylation state and the consequent transposons activation/repression on stress-induced gene expression changes (Chinnusamy and Zhu 2009). *In vitro* cultures are submitted to unusual cultural and environmental conditions (injuries, wounding, high osmoticity, growth regulators treatments etc.) and it is known that they well represent the stress-induced state (Gaspar et al. 2002). Epigenetic changes induced by tissue culture could be expressed as the activation of quiescent loci or as epimutation of loci sensitive to chromatin-level control of expression. In general tissue culture variation is relatively frequent, inherited stably and used to confer useful traits to crop plants; the few cases that do not comply with this feature are unstable variants with particular regard to leaf and flower pigmentation status (Kaeppler et al. 2000).

In somaclonal variants of banana the variegated leaf pattern may be used as a morphological marker for dwarfism (Martin et al. 2006). Leaf variegation in banana plants seems to be related to the endogenous levels of IAA and cytokinins (Zaffari et al. 1998) and consequently it could be attributed to a possible genetic alteration of cytokinins metabolism. In our case the causes of this phenomenon have not been ascertained jet but we cannot exclude the involvement of auxin-cytokinin balance modification as suggested by the rooting pattern of variegated shoots which, opposite of the'BC1', was correlated to callus presence. The fact that cytokinin requirement in cultured plant tissues may be epigenetically regulated by changes in DNA methylation (Meins and Thomas 2003) adds value to our hypothesis. Contrary to that observed for other fruit species (Martin et al. 2006), in our conditions the presence of variegation is not related to any undesirable phenomenon (e.g. dwarfism).

The main concern in the use of antibiotics is their role on morphogenesis. To determine the best antibiotic combination among cefotaxime, vancomycin and timentin which may be used to control the growth of *Agrobacterium* after transformation, we tested their concentration to identify any possible effect on adventitious bud formation. Although vancomycin alone is ineffective against gram negative bacteria it can be utilized in combination with cefotaxime, which creates holes in the lypopolysaccharide envelop, allowing its penetration (Humara and Ordás 1999; Burgos and Alburquerque 2003). In disagreement with previous researches (Nauerby et al. 1997; Humara and Ordás 1999; Burgos and Alburquerque 2003) we found that both timentin (250 mg l^{-1}) and the combination of cefotaxime/vancomycin (200 mg l^{-1} each), although were able to efficiently eliminate *Agrobacterium*, had negative effects on bud induction capacity, reducing the percentage of regeneration compared to the control. Cefotaxime has been used extensively in plant tissue culture, many species have been regenerated in its presence (Nauerby et al. 1997) and in some cases it was even proved to enhance regeneration ability. 200 ppm cefotaxime (James and Dandekar 1991; Yan and Wang 2007) or even lower concentrations (Smigocki and Hammerschlag 1991) have been successfully used in several experiments of *Agrobacterium* mediated transformation. Despite the positive effects of cefotaxime reported in many species, it has been considered phytotoxic for callogenesis in pear when added at the concentration of 200 ppm (Abdollahi et al. 2006) while in birch it was effective only at lower concentrations (Piagnani Valobra and James 1990). Our results confirm the suitability of cefotaxime as decontaminant agent for 'Burlat C1' in terms of non-interference with morphogenesis but its decontamination power in our conditions is still low. Comparing to the preliminary protocol this transformation procedure improved our results as it was possible to get shoot primordia expressing GUS activity. The difficulty to obtain fully transformed shoots is probably due to the complexity of the tissue infected by *Agrobacterium*: morphogenic callus is rich in both, actively proliferating cells and shoot primordia, and in this condition it is easier recovering escapes or chimeric plants. Complexity of morphogenic callus could also explain the difficulty in obtaining decontaminated explants. Morphogenic masses are full of crevices difficult to reach by the antibiotic: this could explain why cefotaxime failed as decontaminant agent even if the concentration used demonstrated to be effective in inhibiting bacterial growth.

Conclusions

A successful method to induce shoot regeneration from somatic explants of the recalcitrant cherry cultivar 'Burlat

C1' is described. Extensive presence of leaf variegation involving *ex novo* regenerated shoots has been here reported, for the first time, in an *in vitro* grown temperate tree fruit. Leaf variegation was not related to any undesired trait and regressed as soon the plantlets were acclimated suggesting that an epigenetic unstable variation had occurred. A relatively wide range of proliferation potential was evidenced among the regenerated shoots which, moreover, showed different rooting performances in respect to the control 'Burlat C1'. According to this the occurrence of stable somaclones need to be better investigated. Among tested antibiotics cefotaxime was proved to be the only able to preserve 'Burlat C1' morphogenic efficiency but, unfortunately, in our conditions it did not show an adequate decontamination power consequently a relatively high number of infected explants could not be successfully decontaminated and were lost during the culture. Nevertheless transient expression was successfully achieved showing that this protocol is not totally ineffective, in fact it is the base on which we are going to better investigate those that demonstrated to be critical aspects: explant type and decontamination phase

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