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A new somaclone of *Prunus avium* shows diverse growth pattern under different spectral quality of radiation

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Abstract

The aim of this research was to set up a regeneration protocol from mature explants of *Prunus avium* L. cv. Hedelfinger and to develop an early screening method for selection of putative somaclones based on morphological and physiological traits regulated by the spectral quality of radiation. DNA analyses of a new somaclone named HS, conducted using Inter Simple Sequence Repeat (ISSR), revealed a polymorphism between the somaclone HS and wild type propagated by microcuttings. When grown under different spectral quality of radiation, somaclone HS showed a different pattern of growth and development compared to the wild type with the main modifications related to apical dominance and chlorophyll production. Somaclone HS showed reduced apical dominance compared to the wild type. Wild type shoots grown in darkness showed chlorophyll *a* and *b* contents at levels in both cases comparable to those recorded under red radiation while HS did not retain the same capability.

Additional key words: apical dominance, branching, cherry, chlorophyll, ISSR markers, polymorphism.

Introduction

Reduction of tree size represents the main goal of the cherry breeding program. Up to now, traditional breeding and agronomic practices, such as dwarfing rootstock and summer pruning, significantly contributed to the reduction of the vegetative growth of cherry tree but these strategies are expensive and require very skilled labour (De Salvador *et al.* 1997). Biotechnology techniques can be considered as an alternative tool to develop programs for improving cherry by using somaclonal variation and genetic transformation. Nevertheless, the use of both requires a suitable regeneration system. Despite several reports on *Prunus* spp., only a few publications referring to regeneration from mature tissues of *Prunus avium* cultivars are available (Yang *et al.* 1991, Hammatt and Grant 1998, Negri *et al.* 1998).

Random genetic variabiiity frequently occurring in cell cultures is preserved among the population of regenerants; this phenomenon was firstly documented for crop plants (Skirvin 1978) and more recently for woody trees (Donovan *et al.* 1994, Harding *et al.* 1996, Muleo *et al.* 1996a, Wang *et al.* 1996, Caboni *et al.* 1999). Nevertheless, very little is understood about how somaclonal variation can occur and be regulated. The event is particularly intriguing because it is presumably under the control of many factors. It has been suggested that tissue culture through simple micropropagation procedure can induce rejuvenation in cherry affecting adventitious roots initiation. A related protein has been isolated in micropropagated plantlets with juvenile phenotypic traits (Hammatt *et al.* 1998).

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Abbreviations: BAP - 6-benzylaminopurine; Chl - chlorophyll; CTAB - hexadecyltrimethyl ammonium bromide; 2,4-D - 2,4-dichlorophenoxyacetic acid; DMF - dimethylformamide; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; TDZ - thidiazuron.
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The aim of the present research was to set up a regeneration protocol for the sweet cherry cultivar Hedelfinger, and to develop an early screening method for selection of putative somaclones which would be based on physiological and morphological traits related to plant growth and development and regulated by the quality of radiation, focusing on the role of photoreceptors for apical dominance and branching control (Ballaré *et al.* 1997). Molecular analyses have been also performed to ascertain the diversity of the somaclone at the genetic level with respect to sequence variation.

Materials and methods

Plants and culture conditions: Axenic shoot cultures of the cherry (*Prunus avium* L.) cv. Hedelfinger (H) were grown in 500 cm^3 transparent boxes (*Green-boxes*, *Duchefa*, Haarlem, The Netherlands) containing 75 cm^3 of solidified medium composed of MS (Murashige and Skoog 1962) minerals, Bourgin and Nitsch (1967) vitamins, 2.5 or 5.0 µM 6-benzylaminopurine (BAP), 3 % (m/v) sucrose, 0.6 % m/v *Gelrite*; pH was adjusted to 5.6 before autoclaving for 15 min at 121 °C. Shoots of the somaclone named HS, originated by direct regeneration from H leaf section as described in the next section, were propagated onto the same medium. Somaclone HS was chosen because it had given some interesting results in a preliminary screening (unpublished data). All cultures were routinely maintained at 22 - 24 °C, under *Philips TDL 33* (Monza, Italy) cool white fluorescent tubes, under irradiance of about 7.5 µmol s^{-1} m^{-2} and 16-h photoperiod and subcultured every 4 weeks on fresh medium.

Shoot rooting was achieved by shoot basal dipping in 50 % ethanolic solution of 1 µg dm^{-3} indole-3-butyric acid (IBA) followed by the culture on growth regulator free half-strength MS medium.

Organogenesis: To induce shoot regeneration, H internodes and roots were cut into small pieces of 0.3 - 0.5 cm while leaves were cut in three transverse strips and placed on the medium with the abaxial surface uppermost. Explants were placed in multiwell dishes (*Sterilin*, Staffordshire, England), each well containing 3 cm^3 agarised medium. Thidiazuron (TDZ; 0, 1.0, 2.0 and 3.0 mg dm^{-3}), was added in different combinations with either indole-3-acetic acid (IAA; 0, 5.0, 0.5 and 1.0 mg dm^{-3}) or 0.4 and 0.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), while BAP (1.0 and 2.0 and 0.4 mg dm^{-3}) was tested, only on leaf explants, in combination with IAA (0.5 and 1.0 mg dm^{-3}) and 2,4-D (0.2 and 0.4 mg dm^{-3}). For each type of explant and for cytokinin per auxin combination minimum four multiwell dishes (100 explants) were evaluated. Data collected were expressed as percentage of explants differentiating callus, roots and shoots.

Radiation quality treatments: Selected shoots, ten for each *Green-box*, were incubated for four weeks under continuous irradiance supplied by different types of lamps, filtered through photoselective layers: *1*) white light as previously described, phytochrome photoequilibrium (Pfr/Ptot) = 0.9 was calculated as elsewhere reported (Muleo and Thomas, 1997); *2*) red radiation obtained with *Philips TDL 18 W/15* fluorescent tubes, irradiance 9 µmol s^{-1} m^{-2}, Pfr/Ptot = 0.75; *3*) blue radiation provided by *Philips TDL 33* cool white fluorescent tubes (filtered through polymethylmethacrylate layers capable of cutting wavelengths shorter than 400 nm and between 650 and 760 nm, irradiance of about 9.8 µmol s^{-1} m^{-2}, Pfr/Ptot = 0.7; *4*) far-red radiation was obtained by filtering radiation provided by *Sy 232* cool tubes (*TecnoLux Italia*, *Desio*, Milan, Italy), spectral output between 700 and 780 nm, through *Spotfilter* polyester layers primary-red (*106*) and dark blue (*119*) (*Simonelli lights*, Milan, Italy), irradiance of 9.7 µmol m^{-2} s^{-1}, Pfr/Ptot = 0.07; *5*) darkness was obtained wrapping each box with two layers of aluminium sheet. For each radiation treatment 3 boxes have been used.

After 4 weeks of culture, the parameters recorded were: number of new axillary buds and shoots, shoot length and the 'distance from the apex' (AD) expressed as the number of nodes included between shoot apex and the first outgrowth lateral bud (Muleo *et al.* 1996b), and chlorophyll (Chl) *a* and *b* contents. Chl was extracted according to Porra *et al.* (1989) from the youngest leaves. Samples of 100 mg each, were immersed overnight in 4 cm^3 of dimethylformamide (DMF) prior to spectrophotometrical determination.

Data were analysed by two-ways *ANOVA* and the differences contrasted using the Tukey's test. Statistical analysis was performed at 5 % level using *Statgraphic* software (*Statistical Graphics Co.*, Rockville, USA). Data expressed in percentage were first subjected to Kolmogorov-Smirnov non parametric test of normality.

DNA analyses: DNA extractions were carried out from leaves of plantlets grown *in vitro*, using hexadecyltrimethyl ammonium bromide (CTAB) as described by Sul and Korban (1996). For the amplification of chloroplast and mitochondrial DNA regions 50 ng of total DNA were submitted to 35 cycles of amplification (1 min 94 °C, 1 min 55 °C, 2 min 72 °C), with an initial melting step (2 min 94 °C), in a total volume of 0.025 cm^3, containing 200 pmol of each primer, 200 µmol of each dNTP, and 0.2 U of Taq polymerase, with the suitable

buffer. For the amplification of total DNA 100 ng of genomic DNA were submitted to 35 cycles of amplification (0.4 min 94 °C, 0.45 min 40 - 45 °C, 1.30 min 72 °C), with an initial melting step (1.5 min 94 °C), in a total volume of 0.025 cm^3 containing the appropriate primers and all the reagents as mentioned before. The sequences of primers used to amplify the cpDNA and mtDNA regions (Demeasure *et al.* 1995), and the sequences of ISSR (Inter Simple Sequence Repeat) primers are shown (Table 1). PCR products were visualised by electrophoresis on 2 - 3 % agarose or 3.5 % acrylamide gels stained with ethidium bromide.

Table 1. Sequence of the primers used in the molecular analyses.

Name	Sequence	Name	Sequence
trn-T	CATTACAAATGCGTATGCTCT	ISSR1	GTGTGTGTGTGTGTYR
trn-D	ACCCATTGAACTACAATCCC	ISSR2	CTCTCTCTCTCTCTCTRA
Mt-1	CAGTGGGTTGGTCTGGTATG	ISSR3	GGCGAGAGAGAGAGAGAGA
Mt-2	TCATATGGGCTACTGAGGAG	ISSR4	CAAGAGAGAGAGA
		ISSR5	TGTGTGTGTGTGYR
Rip	CATTTGTTGTTGTTG	ISSR6	GTGTGTGTGTGTAY
Rip2	CATTGATGATGATGA	ISSR7	CACACACACACARY
Rip3	AGATGAGATGATGA	ISSR8	CACACACACACARG
Ripm	TTGTTGTTGTTG	ISSR9	CTCTCTCTCTCTCTCTRG
Rip430	GAACTAAGAGACC	ISSR10	GTGTGTGTGTGTRG

Results

Organogenesis: Shoot regeneration had quite a discontinuous trend and occurred with very low frequency, in two different ways: *1*) as an early event, observed within 4 - 6 weeks on the inductive medium or *2*) as a late event, observed after 24 and sometimes more weeks, from a complete necrotic callus. In addition, most shoots turned brown and degenerated in a few weeks. Adventitious shoot differentiated from both leaves and roots; nevertheless, only the shoots risen from leaves grew while shoots from roots were weak and usually unable to reach the proliferation stage. Among the different tested auxin and cytokinin combinations those promoting organogenesis were characterised by the presence of 2 mg dm^{-3} TDZ in combination with IAA or 2,4-D (Table 2). 1 % shoot regeneration was recorded for leaf cut cultured on 0.5 mg dm^{-3} IAA; if its concentration was doubled 10 % of explants produced roots. 1 % shoot regeneration was also obtained from root cuts cultured on 0.5 mg dm^{-3} 2,4-D while 20 % of leaf cuts cultured at these conditions differentiated only roots. At the same concentration of 2,4-D, the reduction of TDZ to 1 mg dm^{-3} greatly stimulated root differentiation from root cuts, in fact 90 % of these cuts produced adventitious roots (Table 2). When BAP replaced TDZ in the medium, either with IAA or 2,4-D, organogenesis did not occur and callus formation was observed only at the highest IAA concentration or in the presence of 2,4-D (data not shown).

Table 2. Effect of different concentrations of IAA or 2,4-D and TDZ [mg dm^3] on morphogenesis [%] in leaf (L), internode (I), and root (R) explant culture (n.t.= not tested).

TDZ	IAA			2,4-D	
	0.0	0.5	1.0	0.4	0.5
0.0	0 (L,I)	20 callus (L,I)	20 (L,I)	100 callus (L,I)	100 callus (L,I)
1.0	100 callus (L,I)	100 callus (L)	100 callus (L)	100 callus (L,I)	100 callus (L,I) 90 roots (R)
2.0	0 (L, I)	1 shoots (L) 60 callus (L)	10 roots (L) 100 callus (L)	100 callus (L) 20 roots (L)	1 shoots (R) 20 roots (L)
3.0	n.t.	n.t.	n.t.	30 callus (L)	20 callus (I, L)

Effect of radiation quality on shoot growth: Under white light, the number of new nodes did not differ between H and HS, while the percentage of outgrowth buds was higher in HS than in H. H showed a higher apical dominance compared to HS, in fact, for H shoots the average number of nodes that were inhibited by the apex (AD) was 18.6 while for HS the value was lowered to 12.8 (Table 4). HS shoots grown under blue radiation

showed a peculiar bushy habit due to a poorer main shoot growth (Table 5) and to a relatively high formation of nodes (buds) alongside the stem (Table 3) that, in 30 % of the cases, developed into new shoots (Table 3). Blue radiation contributed to reduction of apical dominance, compared to white light both in the increased percentage of outgrowth buds and diminished apical dominance in the two plant types but on H its effect was stronger (Table 4). Red radiation enhanced main shoot length (Table 5) and reduced node number (Table 3) in both genotypes but once again at a higher degree in H. Regarding the percentage of outgrowth buds to total main shoot stem lateral buds the two genotypes showed an opposite behaviour. Red radiation promoted H and inhibited HS lateral shoot outgrowth (Table 3). Apical dominance was remarkably affected by red radiation only in the case of H (Table 4) while in HS it did not differ from the white light conditions. The responses of H and HS under far-red and dark conditions were quite similar: node number and AD were lowered and the percentage of outgrowth buds and main shoot length were enhanced. In H, light quality effect on shoot length was greater on main shoots than on lateral ones (Table 5); in HS the most noticeable effect on lateral shoot length was due to darkness, in fact shoots grown in dark conditions were nearly twice as much elongated as the control (Table 5).

Table 3. Effect of quality of radiation and genotype on number of nodes formed on elongated leader stem and percentage of outgrowth lateral buds from the total leader stem lateral buds of *in vitro* shoots of wild type (H) and somaclone HS. Means with the same letter are not different at $P = 0.05$ ($n = 20$).

	Nodes [cm^{-1}]		Shoot outgrowth [%]	
	H	HS	H	HS
White	6.9 c	7.3 c	14.6 a	22.4 b
Blue	7.0 c	9.1 d	27.9 bc	30.0 cd
Red	3.8 a	5.7 b	22.4 b	16.0 a
Far-red	3.5 a	4.1 a	32.8 cd	35.2 cd
Darkness	4.3 a	4.7 a	37.8 d	31.2 bc

Table 4. Effect of quality of radiation and genotype on number of nodes between shoot apex and the first proximal lateral outgrowth shoot or disclosed bud (distance from apex) on *in vitro* shoots of wild type (H) and somaclone HS. Means with the same letter are not different at $P = 0.05$ ($n = 20$).

	Distance from apex	
	H	HS
White	18.6 e	12.8 d
Blue	13.0 d	9.8 bc
Red	11.9 cd	12.1 cd
Far-red	5.6 a	5.1 a
Darkness	8.5 b	6.9 ab

Table 5. Effect of quality of radiation and genotype interaction on main shoot length and axillary shoots mean length [cm] in wild type (H) and somaclone HS. Means with the same letter are not different at $P = 0.05$ ($n = 20$).

	Main shoot length [cm]		Lateral shoot length [cm]	
	H	HS	H	HS
White	2.7 b	2.5 b	0.10 a	0.46 bc
Blue	2.4 b	1.9 a	0.19 a	0.28 abc
Red	3.7 d	2.8 bc	0.31 abc	0.44 bc
Far-red	3.3 cd	3.2 cd	0.20 ab	0.48 c
Darkness	3.0 bcd	2.8 bc	0.20 ab	0.80 d

Chlorophyll content of newly formed leaves: Chl *a* content under white and blue radiation was not significantly different in the two genotypes (Table 6). Red radiation slightly lowered the content only in the case of H, while under far-red it dropped nearly to zero both in H and HS. H shoots grown in darkness showed Chl *a* and *b* content at levels in both cases comparable to those recorded under red radiation (Table 6) while HS did not retain the same capability. Red and, at a higher degree far-red radiation, affected Chl *b* concentration in both plant types while blue radiation significantly enhanced HS Chl *b*, this leading to score the highest total Chl amount recorded (Table 6).

Table 6. Chlorophyll *a* and *b* content in new leaves: comparison between H and somaclone HS shoots grown under different quality of radiation. Means with the same letter are not different according at $P = 0.05$ ($n = 20$).

	Chl *a* [mg g^{-1}(d.m.)]		Chl *b* [mg g^{-1}(d.m.)]	
	H	HS	H	HS
White	17.2 c	18.0 c	10.4 c	9.6 c
Blue	17.3 c	19.3 c	10.4 c	13.4 d
Red	13.6 b	18.6 c	4.2 b	6.7 b
Far-red	0.8 a	0.5 a	0.2 a	0.2 a
Darkness	12.5 b	1.2 a	5.0 b	1.5 a

Table 7. Chlorophyll *a+b* content in new leaves: comparison between H and somaclone HS shoots grown under different quality of radiation. Means with the same letter are not different according at $P = 0.05$ ($n = 20$).

	Chl *a+b* [mg g^{-1} (d.m.)]	
	H	HS
White	27.6 d	27.6 d
Blue	27.7 d	31.9 e
Red	17.8 b	24.5 c
Far-red	1.0 a	0.7 a
Darkness	17.5 b	2.7 a

DNA analyses: DNA analysis of non-coding region of chloroplast DNA (trn) and mitochondrial DNA, NADreductase gene region, no differences between H and HS were detected (data not shown). A total of 15 ISSR primers were screened for the amplification of genomic DNA, each amplification experiment being carried out three times to ensure the reliability of the technique, and only reproducible fragments were considered. ISSR5 primer did not amplify any DNA fragment, while amplifications profiles obtained through

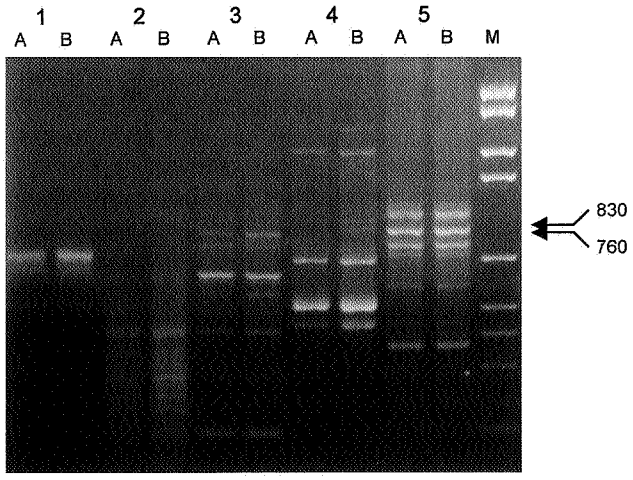

Fig. 1. ISSR amplification patterns as visualised on 1.5 % agarose gel. *A* - H (wild type), *B* - HS (somaclone), and *M* - Marker VI. Numbers referred to ISSR primers used: 1 - ISSR2, 2 - ISSR3, 3 - ISSR4, 4 - ISSR6, 5 - ISSR7. The *lateral arrows* indicate the position and size of polymorphic fragments present in HS.

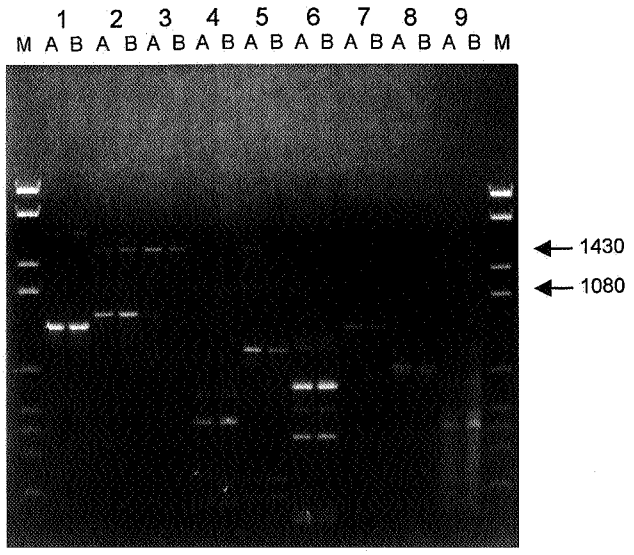

Fig. 2. ISSR amplification patterns as visualised on 1.5 % agarose gel. *A* - H (wild type), *B* - HS (somaclone), and M - Marker VI. Numbers referred to ISSR primers used: 1 - RIP, 2 - RIP2, 3 - RIP3, 4 - ISSR10, 5 - ISSR8, 6 - ISSR9, 7 - ISSR1, 8 - ISSR2, 9 - ISSR3. The *lateral arrows* indicate the position and size of polymorphic fragments present in H.

ISSR6 and ISSR8 primers were able to reveal polymorphism between H and HS (Figs. 1 and 2): 2 fragments of about 830 and 760 bp are present in HS, and 2 fragments of about 1430 and 1080 bp are present in H. Out of a total number of 80 analysed fragments 4 were polymorphic.

Discussion

P. avium cv. Hedelfinger is a recalcitrant genotype as indicated by the very poor regeneration frequencies. Nevertheless, TDZ in combination with IAA or 2,4-D was the most effective. This agrees with the results of previous research on regeneration from leaves of cherry rootstocks (Hammatt and Grant 1998). Explant source should be also taken in account when performing a regeneration trial: in our conditions, adventitious shoots differentiated from leaves showed higher chances to survive compared to those derived from root cuts.

Somaclonal can affect plants regenerated from cell and tissue culture. We have found considerable differences in growth and development responses between cv. Hedelfinger and somaclone HS, which are probably regulated by different responses to quality of radiation. Differently from data reported for other species of *Prunus* (Muleo and Thomas 1997) in our conditions the weakest apical dominance was recorded in darkness or under far-red radiation, the strongest under "white light", while blue and red radiation had an intermediate effect. The quality of radiation has played a different role on Chl *a* and Chl *b* contents in somaclone HS and H. In particular, blue radiation significantly promoted Chl *b* accumulation only in somaclone HS. Photoreceptors regulate the expression, partially at the level of transcription, of multigenic families encoding Chl *a* and *b* binding proteins, further molecular investigations may indicate the role of such genes in the changes observed in the regenerated shoots.

H is able to accumulate chlorophyll in the dark at the same level as that reached under red radiation. This is not surprising considering that angiosperms, like algae and gymnosperms, can also synthesise chlorophyll via a light-independent route which, however, needs light to be activated (Adamson *et al.* 1997). On the contrary the somaclone HS was not able to retain the same capability when grown in the dark, suggesting some change causing the failure of the light independent pathway or the enhancement of Chl breakdown (Walmsley and Adamson 1994).

The results obtained with the molecular markers have unambiguously proved that the somaclone HS is different from wild type H, although no difference were found in mtDNA and cpDNA. ISSR marker assay has been shown to be useful to evidence variable regions of DNA (Kantety *et al.* 1995), and it has been used in genetic fingerprinting and diversity analysis in rice and maize and various other plant and animals (Zietkienicz *et al.* 1994, Salimath *et al.* 1995, Godwin *et al.* 1997, Parsons *et al.* 1997, Reddy *et al.* 1999). In the present study, ISSR assay was able to differentiate the somaclone HS from the wild type genotype. In fact, as pointed out in the results, there are specific polymorphic bands (5 % of total) which are able to characterise each genotypes.

The study of somaclone HS, under *in vivo* conditions, could provide insight into both the role of the interaction between light quality and hormones in controlling apical dominance and chlorophyll synthesis in woody plants.

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