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Ethylene Inhibitor silver nitrate enhances regeneration and genetic transformation of *Prunus avium* (L.) cv Stella. --Manuscript Draft--

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Abstract:	<p>The effects of the ethylene inhibitors, silver nitrate (AgNO₃) and aminoethoxyvinylglycine (AVG), together with Agrobacterium co-cultivation system were investigated for improving both regeneration and genetic transformation of leaf explants of the recalcitrant sweet cherry cultivar Stella. Co-cultivated young leaf explants, with <i>A. tumefaciens</i> strain EHA105, harbouring a binary vector pROK containing both the phytochrome B of <i>A. thaliana</i> and the nptII genes, were incubated on medium supplemented with either AVG or AgNO₃ for 21 days in the dark, and then transferred onto ethylene-free inhibitors medium in the light. Efficiency in terms of frequency on shoot organogenesis was observed in both explants subjected to co-cultivation with Agrobacterium and not co-cultivated in presence of each ethylene inhibitors. Ethylene inhibitors helped in the morphogenesis process and mass production of shoots. An increase of the regeneration efficiency was observed in ethylene-inhibitor-free medium, which was almost 4 times greater than the control leaf explants. When ethylene inhibitors were added to the medium, a further regeneration efficiency enhancement for AVG (45%) and AgNO₃ (63.3%) was observed. Whereas, in explants not co-cultivated with Agrobacterium, the resulting regeneration efficiency was 25% for AVG and 23.3%, for AgNO₃. Furthermore the regeneration frequency from leaf explants was also enhanced in treatments with aminoethoxyvinylglycine. AgNO₃ also contributed to control Agrobacterium growth in the post-co-cultivation phase. Only the lines originated from AgNO₃-treated explants resulted showed kanamycin resistance, and PCR analysis confirmed both the insertion of the alien genes into the plant genome and the absence of bacterial contamination of the transgenic shoots.</p>
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1 **Ethylene Inhibitor silver nitrate enhances regeneration and**
2 **genetic transformation of *Prunus avium* (L.) cv Stella.**

3

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10 Key Message: High regeneration and transformation efficiency in sweet cherry.

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30 *phytochrome B*, transgenic plant.

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33 **Abstract**

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35 (AVG), together with *Agrobacterium* co-cultivation system were investigated for improving both
36 regeneration and genetic transformation of leaf explants of the recalcitrant sweet cherry cultivar Stella.
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38 containing both the *phytochrome B* of *A. thaliana* and the *nptII* genes, were incubated on medium
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42 ethylene inhibitors. Ethylene inhibitors helped in the morphogenesis process and mass production of
43 shoots. An increase of the regeneration efficiency was observed in ethylene-inhibitor-free medium, which
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45 medium, a further regeneration efficiency enhancement for AVG (45%) and AgNO₃ (63.3%) was
46 observed. Whereas, in explants not co-cultivated with *Agrobacterium*, the resulting regeneration
47 efficiency was 25% for AVG and 23.3%, for AgNO₃. Furthermore the regeneration frequency from leaf
48 explants was also enhanced in treatments with aminoethoxyvinylglycine. AgNO₃ also contributed to
49 control *Agrobacterium* growth in the post-co-cultivation phase. Only the lines originated from AgNO₃-
50 treated explants resulted showed kanamycin resistance, and PCR analysis confirmed both the insertion of
51 the alien genes into the plant genome and the absence of bacterial contamination of the transgenic shoots.

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68 **Introduction**

69 Sweet cherry (*Prunus avium* L.) is an important cultivated fruit tree species in many temperate
70 countries. One of the main objectives in the breeding programmes involving this plant is controlling some
71 of the tree traits, in particular reducing the tree size. Conventional breeding programmes of cherry
72 cultivars and rootstocks are difficult to carry out due to the plant's self-incompatible nature. In addition,
73 when cultivars are grafted on semi-dwarfing rootstocks they do not fully show size reduction and the
74 traits tend to have a chimerical nature and often get lost in subsequent propagation cycles (Sansavini and
75 Lugli 2008).

76 The ability of single plant to modulate the developmental plasticity of its canopy will confer high
77 fitness under specific surrounding of light quality environments (Schmitt et al. 2003). Shade avoidance is
78 considered as the plant response to light quality stress conditions that interferes with the normal
79 development (Franklin and Whitelam 2004). Light quality is strongly modified, not only under the natural
80 heavy canopy of the orchard, but also by the light scattered or emitted by neighbouring plants, influencing
81 the rate of plant development (Gilbert et al. 2001). Perception of light relatively enriched in far-red
82 wavelengths, occurs through the phytochrome photoreceptor system and leads to adaptive plasticity
83 (Mathews 2005). In shoots of *Prunus cerasifera* L. cultured *in vitro*, modular development, apical
84 dominance and bud release were differently regulated by light qualities (Muleo et al. 2001; Muleo and
85 Thomas 1997). Taking into account all these light quality effects we hypothesized that an over-expression
86 of phytochrome may differently modulate plant canopy development in *Prunus avium* L..

87 Genetic engineering techniques are a tool for transferring specific genes that affect plant
88 architecture to the genome of commercial sweet cherry cultivars (Holefors et al. 1998; Holefors et al.
89 2000). Since sweet cherry is known as a recalcitrant woody species, a highly efficient, reproducible and
90 quick shoot regeneration methodology is a prerequisite to have both success after genetic transformation
91 experiments and to avoid abnormal regenerants. Despite several reports on *Prunus* spp., only a few
92 publications regarding the regeneration from mature tissues of *P. avium* cultivars are available (Bhagwat
93 and Lane 2004; Canli and Tian 2008; Druart 1980; Feeney et al. 2007; Gutierrez and Rugini 2004;
94 Gutierrez-Pesce et al. 1998; Lane and Cossio 1986; Oh et al. 1991; Piagnani et al. 2002; Piagnani and
95 Chiozzotto 2010; Tang et al. 2002; Yang et al. 1991). Also, as for sweet cherry, both regeneration
96 efficiency and *Agrobacterium* transformation are genotype dependent, making a cultivar based
97 transformation protocol necessary (Druart et al. 1998; Piagnani and Chiozzotto 2010).

98 Ethylene is produced during *in vitro* culture inside airtight vessels up to a physiological
99 threshold. Accumulation of this gas is associated with poor regeneration and reduced gene transfer
100 efficiency (Seong et al. 2005). Researchers have attempted to limit the effect of ethylene during
101 regeneration using two strategies: by eliminating the gas through forced ventilation, or by using chemical
102 compounds either to inhibit its synthesis or to contrast the hormone action (Arigita et al. 2003). The
103 addition to the medium of silver ions in the form of nitrate, such as Silver Nitrate (AgNO_3), an inhibitor
104 of ethylene action, has shown to be effective in enhancing regeneration, and therefore transformation
105 probability, having an important role in influencing somatic embryogenesis and shoot formation in many
106 *in vitro* plant tissues (Kumar et al. 2009; Wang and Xu 2008). On the other hand, the effect of the

107 aminoethoxyvinylglycine (AVG), an inhibitor of ethylene synthesis, appears to be controversial, reducing
108 shoot regeneration but increasing transformation rate through *Agrobacterium* transformation (Ezura et al.
109 2000; Seong et al. 2005).

110 The objective of this work was to develop both an efficient *in vitro* regeneration method and a
111 genetic transformation protocol for the sweet cherry cultivar Stella, by studying the influence of AVG and
112 AgNO₃, in order to obtain transgenic plants over-expressing *Arabidopsis thaliana phytochrome B gene*
113 (*AtphyB*).

114

115 **Materials and methods**

116

117 Plant and culture conditions, inhibitor solutions, bacterial strain and transformation

118

119 Axenic shoot cultures from cherry cv Stella were grown on a modified MS medium (Murashige
120 and Skoog 1962) called M1, consisting of half concentration of Ammonium Nitrate (NH₄NO₃), and
121 supplemented with 6-Benzylaminopurine (BAP) (1.5 mg l⁻¹), Indole-3-butyric acid (IBA) (0.01 mg l⁻¹),
122 Giberellic Acid (GA₃) (0.1 mg l⁻¹) and 3% (w/v) of sucrose. pH was adjusted to 5.8 before adding 6 g l⁻¹
123 agar (Duchefa, NL) and 8 g l⁻¹ of pectin. All explants were subcultured every twenty days in fresh
124 medium and kept at 24° C ± 1° under a 16 h photoperiod at an irradiance of 40 μmol m⁻² s⁻¹.

125 For shoot regeneration from leaf explants M1 medium was modified adding the growth
126 regulators Thidiazuron (TDZ) (2 mg l⁻¹), which resulted in efficient *P. cerasus* regeneration (Song and
127 Sink 2005), and Indole-3-acetic acid (IAA) (0.5 mg l⁻¹) and is subsequently referred to as M2 medium.

128 Silver Nitrate (AgNO₃) was dissolved in distilled water, at a concentration of 50 mg l⁻¹ and kept
129 at 4° C protected from the light. AVG was dissolved in distilled water at a concentration of 0.5 mg ml⁻¹
130 and kept at -20° C. Each chemical inhibitor was filter-sterilized and added into the appropriate medium
131 after autoclaving.

132 *Agrobacterium tumefaciens* strain EHA 105, with the pTiBo542 plasmid and the binary vector
133 pROK2 was used for transformation. The vector, harboured the neomycin phosphotransferase gene
134 (*nptII*) directed by the *nos* promoter and the cDNA of *AtphyB* (AY466496) driven by the CaMV 35S
135 promoter. The vector was introduced into *Agrobacterium* EHA 105 using freeze-thaw transformation as
136 described by Holefors et al. (2000). For the transformation experiments, single colonies were cultured in
137 10 ml liquid LB medium supplemented by 100 mg l⁻¹ kanamycin at 28° C in dark overnight with rotary
138 shaking (80 rpm). The cells were then collected by centrifugation at 2500 x g for 10 minutes. The pellet
139 was re-suspended in MS liquid medium with 3% (w/v) sucrose to a final OD₆₀₀ of 0.3.

140

141 Shoot regeneration response with AVG and AgNO₃

142

143 To assess the optimum amount of ethylene inhibitors to add onto the medium M2 during the
144 regeneration experiments, two concentrations of AVG (0.5 and 1 mg l⁻¹) and AgNO₃ (50, 100 mg l⁻¹)
145 were tested against the control.

146 The third and fourth apical young expanded leaves, from 15-days old proliferating shoots, were
147 used in regeneration trials. Every leaf was cut transversely four times across the midrib without separating
148 the portions and placed with the adaxial side on the surface of regenerating medium. For each
149 experimental condition, regeneration efficiency was measured in six flasks containing 10 leaf explants
150 each. All experiments were repeated three times.

151

152 Ethylene measurements

153

154 The same leaf explants used for the shoot regeneration analyses were used for monitoring
155 ethylene accumulation. The 100 ml flasks containing the leaf explants were hermetically closed and
156 opened every 24 hours for 4 consecutive days to allow air exchange and avoid accumulation of ethylene
157 produced during wounding.

158 Ethylene was monitored in 1 ml air sample during the regeneration process. Samples were taken
159 using a sterile syringe via a seal in the lid of the flask. Gas analysis was performed by injecting 1 ml of air
160 sample into a Carlo Erba Fractovap 4200 (Carlo Erba Spa, Milano, Italy) gas chromatograph, equipped
161 with a 1-m alumina column (80-100 mesh) and a flame ionisation detector. The detector temperature was
162 100° C. Ethylene accumulation ($\mu\text{l g}^{-1} \text{h}^{-1}$) was then recorded every 24 hours for 5 times. Six replications
163 were considered per treatment, and the experiment was repeated three times.

164

165 Effect of AgNO_3 on bacterial growth

166

167 To assess how different AgNO_3 concentrations affect *A. tumefaciens* growth rate, bacterium cells
168 were grown in Luria–Bertani (LB) liquid medium until the late exponential phase, centrifuged at 2500
169 rpm and resuspended in 20 ml LB medium to reach a final OD_{600} concentration of 0.3. Aliquots of this
170 culture (10 μl) were plated onto into 6-well polystyrene (35 mm \varnothing) flat-bottom microplates containing 5
171 ml of LB solid medium enriched with kanamycin and different AgNO_3 concentrations (0, 2, 4, 6, 8, 10
172 mg l^{-1}). The inoculated microplates were incubated for four days at 28° C in the dark. Bacterium colony
173 growth rates were estimated by visual measurement of the diameter of the colonies. The experiments
174 were done in triplicate.

175

176 Cherry cv Stella shoots' kanamycin tolerance

177

178 Cherry cv Stella natural kanamycin resistance was tested in a preliminary experiment in order to
179 determine the appropriate concentration to add into the medium as selective agent of the transgenic lines.
180 Untransformed *in vitro* shoots originating from leaf explants were transferred onto M1 medium
181 containing five different kanamycin concentrations (0, 10, 20, 30, 40, 50 mg l^{-1}). Three glass jars
182 containing 100 ml of medium M1 and ten uniform shoots were used for each culture condition and
183 incubated as previously described. After a four-week culture period, the percentage of surviving shoots
184 was calculated. The experiment was repeated twice.

185

186 Shoot induction and transformation procedure

187

188 To test the possible interaction between AgNO₃, AVG and *Agrobacterium* during co-culture of
189 young expanded leaves two main treatments were conducted. In all regeneration experiments, leaf
190 explants from plantlets grown in M1 medium, and cut as previously described, were incubated abaxial
191 side up on M2 media for two days in the dark.

192 Before moving the explants to M2 medium, half of the leaf explants were co-cultivated for 2 hrs
193 in MS liquid basal medium with *A. tumefaciens* (A), while the other half was cultivated in the same
194 bacterial-free medium (B) as control.

195 After two days on M2, each group of explants was divided in three sub-groups. Each sub-group
196 was cultivated on M2 medium without ethylene inhibitors (0) as a control, or M2 medium containing
197 AVG (I) or AgNO₃ (II) at 0.5 mg l⁻¹ and 50 mg l⁻¹, respectively.

198 Cefotaxime (200 mg l⁻¹) was added to all media, to eliminate *Agrobacterium*. Explants were
199 incubated for 21 days in the dark, and then transferred onto free ethylene inhibitors M2 medium, under 16
200 h photoperiod, at an irradiance of 40 μmol m⁻² s⁻¹. After ten days small green shoots were selected with
201 the aid of a stereoscope and moved on M1 containing 10 mg l⁻¹ kanamycin, under the same photoperiod
202 as described above. Final selection was carried out onto M1 medium enriched with 50 mg l⁻¹ of
203 kanamycin, subculturing every 7 days. The number of explants regenerating shoots (regeneration
204 efficiency) and the number of regenerated shoots per explant (regeneration frequency) were recorded after
205 28 days. Each treatment consisted of 6 Petri dishes, each containing ten leaves. All experiments were
206 repeated twice with reproducible effects; data from all trials were pooled together for the statistical
207 analysis.

208 Regeneration efficiency data were analysed as percentage, transformed by the arcsine square root, and on
209 all data of both parameters were subject to analysis of variance (ANOVA) at 5% level of significance
210 (P=0.05), and mean separation was done by Tukey's test as an All Pairwise Multiple Comparison
211 Procedure, using the SigmaStat 3.1 package (SYSTAT software Inc., Chicago, IL, USA).

212

213 Plant, bacterial and plasmid DNA extractions, and plant mRNA extraction

214

215 DNA was extracted from 100 mg leafy shoot tissues collected from three-week old putative
216 transgenic and wild type control lines, using Plant DNAzol Kit (Life Technologies, Molecular Research
217 Center Inc, Cincinnati, OH), following the manufacturers' guidelines. Three independent DNA
218 extractions were performed for each line. Genomic and plasmidic DNA from *A. tumefaciens* strain
219 EHA105 overnight culture was extracted as described by Ausubel and et al., (1992). Messenger RNA was
220 extracted from three *in vitro* plantlets of each transgenic line, using Nucleo Spin RNA plant (Macherey-
221 Nagel, Germany) protocol, following the manufacturers' guidelines. DNA and RNA quantifications were
222 done according to Sambrook et al., (1989).

223 Ready-to-go RT-PCR beads Kit (Amersham, UK) was used for the retro-transcription of mRNA
224 into cDNA, using oligo-dT primers, following the manufacturers' guideline. The profile of retro-
225 transcription was 42° C per 30 min and 95° per 5 min.

226

227 Plant and bacterial genes primers design

228

229 Based on the assumption that genes encoding for the same proteins in different species show
230 conserved domain with high degree of identity, divergent regions between cherry *phyB* gene and *AtphyB*
231 were located. The selected regions were checked against bacteria genes as well and it did not match any
232 homologous of eukaryotic sequences of genes present in data bank. *AtphyB* specific primers were
233 therefore designed in the selected regions (Online Resource 1).

234 PCRs were carried out using specific primers to test for the presence of the *AtphyB*, *nptII*, and
235 *Agrobacterium trpR* genes (Online Resource 2). PCR mixes comprised 1 µl from the cDNA synthesis
236 reaction or 1 µl from a touch from a single bacterial colony dispensed in 40 µl SDW, 0.4 U KOD Hot
237 Start DNA Polymerase (Merck Chemicals, Cat. No. 71086), 1X KOD Hot Start DNA Polymerase
238 reaction buffer, 2 mM MgSO₄, 0.2 mM dNTPs and 0.5 µM of each forward and reverse primer in a total
239 volume of 20 µl made up with SDW. The same reaction, but in a volume of 10 µl was made for gDNA
240 screens using 0.5 µl of template. The amplification consisted of an initial denaturation at 94° C for 2 min,
241 followed by 30 cycles (if not specified otherwise) of denaturation at 94° C for 15 s, annealing (specific
242 primer temperature) for 30 s, and extension at 72° C for 1 min per kb of expected product. A further 10
243 minutes of extension at 72° C was carried out at the end of the cycles. Primers sequences, expected
244 product sizes and annealing temperatures used to detect each gene are presented in Online Resource 2.
245 Amplification products were visualised on agarose gels (1.2%, w/v) and ethidium bromide stained (10
246 µg/ml).

247

248 **Results**

249

250 Effect of AVG and AgNO₃ on ethylene accumulation and shoot regeneration

251

252 Ethylene daily rate accumulation was measured in vessels where leaf explants were regenerated
253 on medium containing different concentration of AVG and AgNO₃ (Fig. 1).

254 AVG strongly reduced the production of ethylene and had a strong effect on regeneration (Fig.
255 1a). Ethylene accumulation was reduced by half in the 0.5 mg l⁻¹ AVG treatment, and dropped to zero
256 when the inhibitor concentration was increased to 1 mg l⁻¹ (Fig. 1a). The highest regeneration efficiency
257 (25±2.2 %) was induced with 0.5 mg l⁻¹ of AVG in the medium, while at higher concentration, a strong
258 decrement in regeneration efficiency occurred (5±1.5 %) (Fig. 1a). For this reasons in the following
259 experiments 0.5 mg l⁻¹ of AVG was selected for enriching the M2 medium.

260

261 As expected, AgNO₃ did not have an effect on ethylene synthesis and the daily accumulation was
not blocked (Fig. 1a). But the increase in AgNO₃ concentration in the M2 medium matched with an

262 enhanced regeneration efficiency moving from 8.3 ± 3.1 to 23.3 ± 2.2 and 25.5 ± 3.5 % adding 0, 50 and 100
263 mg l^{-1} , respectively to the medium M2 (Fig. 1b). The lowest amount of AgNO_3 was selected for enriching
264 the M2 medium in the subsequent experiments.

265

266 Effect of kanamycin on shoot regeneration and of AgNO_3 on *Agrobacterium* growth

267

268 Preliminary experiments were conducted to establish the correct kanamycin concentration to
269 discriminate putative transgenic plants. Kanamycin inhibited shoots growth by inducing yellowing of
270 leaves and apices from the concentration of 30 mg l^{-1} (Fig. 2a). At higher kanamycin concentrations the
271 shoots showed atrophied development or they died. Although 30 mg l^{-1} of kanamycin was originally
272 judged to give good discrimination between transformed and untransformed tissues, the concentration of
273 50 mg l^{-1} was used in all subsequent experiments in order to avoid unexpected 'escape' plants.

274 As previous works in our lab showed that false positive transgenic plants could survive on
275 selective medium due to the presence of *Agrobacterium* in the plant tissue, the antibacterial action of
276 AgNO_3 at different concentration was used as further selector. No *Agrobacterium* growth was observed at
277 the two highest concentrations tested, 8 and 10 mg l^{-1} of AgNO_3 , during the ten days incubation period
278 (Fig. 2b).

279

280 Effect of AgNO_3 and AVG and synergistic effect with *Agrobacterium* on regeneration

281

282 The first regeneration events were visible on leaf explants after two weeks of culture in dark
283 conditions. Small leafy buds developed directly on the cut edges and midribs of leaf tissue. Elongated
284 microshoots evolved from buds were visible during the 4th week of culture. Both treatments AVG (AI)
285 and AgNO_3 (AII) increased the regeneration efficiency, roughly to 25% and 23%, respectively, compared
286 to the untreated leaf explants 8.3% (A0) (Fig. 3). When leaf explants were co-cultivated with
287 *Agrobacterium*, the regeneration efficiency increased to 45% in presence AVG BI-treatment, and 63.3%
288 in AgNO_3 BII-treatment. Comparable results were found in the three repeated experiments.

289 *Agrobacterium*, AVG and AgNO_3 also increased the regeneration frequency. In BI and BII
290 treatments, the frequency of neo-developed shoots was high, compared to the control and many of those
291 shoots were kanamycin resistant during the selection procedures. Explants co-cultivated and AVG-treated
292 gave the highest induction, producing around 5.3 shoots per explants (Table 1).

293

294 Effect of AgNO_3 and AVG on transformation and molecular analysis of transgenic plants

295

296 Transformation experiments produced green kanamycin resistant shoots from leaf explants
297 treated with AVG and AgNO_3 . Shoots were transferred to M1 medium, containing kanamycin to select
298 putative transgenic lines, and subcultured for four/five times for rapid and clonal multiplication. When
299 shoots were tested by PCR analysis, only 9 lines out of 28 from AgNO_3 treated leaf explants produced the

300 expected sizes fragments *phyB* gene (Online Resource 3b) (Table 1). No transgenic shoots were detected
301 by PCR analyses among the lines from AVG treated leaf explants (Table 1).

302 To test the presence of any bacterial contamination in the plant tissue, further PCR analyses were
303 conducted using specific primers for *trpR* bacterial gene. Previously, *trpR* primers were experimented in
304 other transgenic species obtained in our laboratory in order to assess their effectiveness. No amplicons
305 were produced from transgenic and WT plant DNA of the species tested (Online Resource 3a). No
306 amplicons were also observed in the 9 transgenic lines and in the WT of cherry (Online Resource 3a).

307

308 **Discussion**

309

310 This study aimed to develop an appropriate and reproducible protocol for *in vitro* regeneration
311 and transformation of sweet cherry cv. Stella, which is recalcitrant to *in vitro* culture. It has been shown
312 that the regeneration efficiency of sweet cherry is genotype dependent and a very large range of
313 regeneration efficiency has been reported in different cultivars (between 1 and 70%) (Bhagwat and Lane
314 2004; Canli and Tian 2008; Matt and Jehle 2005; Piagnani et al. 2002; Piagnani and Chiozzotto 2010).
315 Using silver thiosulfate as ethylene inhibitor, previous work on *P. avium* on 5 different cultivars, namely
316 Schneiders, Sweet-heart, Starking Hardy Giant, Kordia and Regina, found that the regeneration efficiency
317 was reduced in four of of the five sweet cherry cultivars (Matt and Jehle 2005). The regeneration
318 efficiency was only increased in the cv Sweetheart (31.2%) when 0.02 M of silver thiosulfate was
319 included in the medium (Matt and Jehle 2005). Regeneration efficiency proved to be cultivar specific and
320 only few transgenic plants have been generated and are currently present in agriculture (Cheong 2012). In
321 our work, we present a protocol that increases regeneration events 3-fold in *P. avium* cv Stella with and
322 without co-cultivation with *Agrobacterium*.

323

324 Several authors have reported a beneficial effect of AVG on morphogenesis, stimulating the
325 formation of adventitious roots in wild cherry and enhancing, *in vitro*, root formation in apple shoots
326 (Biondi et al. 1990; Ma et al. 1998). However, AVG was also reported to inhibit somatic embryo
327 formation in *Medicago sativa*, even at very low concentrations (Meijer and Brown 1988). These findings
328 suggest that a low ethylene amount might be required in regeneration (Kumar et al. 1996). AgNO₃
329 promoted regeneration of roses and pomegranate and induced or improved regeneration in apricot and
330 plum explants (Escalettes and Dosba 1993; Naik and Chand 2003; Orlikowska et al. 1996). In our
331 experiments both ethylene inhibitors used improved, alone and in synergistic action with *Agrobacterium*,
332 the regeneration efficiency as well as the number of regenerative events. However, when AVG was added
333 to the medium at highest amount the daily rate of ethylene production was almost null and the
334 regeneration efficiency dropped dramatically. Therefore a low-threshold of ethylene may be necessary to
335 induce shoot regeneration. This hypothesis seems to be confirmed in lettuce, where transgenic lines
336 ethylene insensitive over-expressing the *etr1-1* gene showed an altered pattern of regeneration, with an
inhibition of shoots formation and a stimulated root formation (Kim and Botella, 2004).

337

338 The effects of *Agrobacterium* on plant cell dedifferentiation and shoot regeneration as well as the
cross-talk system, between bacteria and tissue plant, are not completely understood. Much is known about

339 *vir* complex genes and DNA delivery and insertion; however, there is little knowledge on induction and
340 development of regeneration events (Pitzschke and Hirt 2010). According to our results, *Agrobacterium*
341 could synthesize, or induce, the synthesis of compounds capable to enhance regeneration in cherry. This
342 effect is further enhanced when explants are subsequently cultured in media enriched with ethylene
343 inhibitors, showing a synergistic effect.

344 Our results also suggest that ethylene synthesis is important for gene insertion, as it is proved by
345 the stable development and survival of transgenic lines. In broccoli, transformation efficiency was
346 enhanced when tissues were transformed with a construct bearing an antisense ACC oxidase gene
347 (Gapper et al. 2002). In our study, we showed that low level of ethylene synthesis and reduction of
348 ethylene sensitivity induced by silver nitrate is important for regeneration and genetic transformation.
349 These results confirm previous work conducted in apple where AgNO_3 increased transformation
350 efficiency (Seong et al. 2005).

351 Orlikowska et al. (1996), found that silver nitrate retarded *Agrobacterium* growth on
352 regeneration media used for roses but they also reported not differences in bacterial growth on LB
353 medium enriched by silver nitrate. Our results showed that *Agrobacterium* growth is repressed by AgNO_3
354 with a toxic effect for the organism at the dosage used in the M2 medium (50 mg l^{-1}). In fact, during post
355 *Agrobacterium* co-cultivation where only cefotaxime (200 mg/L) was used for multiplication and
356 elongation phase, inefficient control of bacteria growth was observed after some months as also observed
357 by Mendoza-de Gyves et al. (2010).

358 Transgenic plants of cv Stella bearing the heterologous *phyB* gene were produced using AgNO_3
359 added to the induction medium, suggesting that ethylene may be involved in two different ways on
360 regeneration induction and transformation event. The increase in regeneration indicates that both reducing
361 the production and changing the perception of ethylene, promote cell differentiation and shoot
362 organogenesis in leaf explant tissues. Adding AgNO_3 to the medium changes the plants sensitivity to
363 ethylene without modifying ethylene production. This resulted in a stable gene insertion in the plants,
364 probably because plant cells become less sensitive to the adverse effects of *A. tumefaciens* infection.
365 Ethylene is strongly implicated in the interaction between plant and plant pathogens. In tomato, ethylene-
366 insensitive and ethylene-under producing mutants showed tolerance to the pathogen *Xanthomonas*
367 *campestris* with reduced symptoms to the infection (O'Donnell et al. 2001).

368 The non-amplification of *trpR* fragment, also indicate the absence of bacterial contamination in
369 plant tissues of cv Stella transgenic lines, indicating that the alien gene is stably inserted in the genome
370 and providing an early and efficient diagnostic test for transformation.

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381

382 **Author contributions**

383 T.S., B.T. and R.M. designed the experiments. T.S. performed the experiments. T.S., B.T. and R.M. wrote
384 the manuscript.

385

386 **Electronic supplementary material**

387 The online version of this article contains supplementary material, which is available to authorized users.

388

389 **Competing financial interests:** The authors declare no competing financial interests.

390

391

392 **References**

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 496

497 Table 1: Effect of AVG (I) and AgNO₃ (II) applied after co-cultivation with *Agrobacterium* (A) or alone
 498 (B) or on the regeneration frequency (number of regenerated shoots per regenerating explant) and
 499 transformation efficiency, as recorded in the 3rd trial. Sixty explants were used for each treatment. Means
 500 within a column followed by the same letter are not significantly different by Tukey's test (P<0.05).
 501

Treatments	Total regenerating explants	Total regenerated shoots	Regeneration frequency	Total lines kanamycin resistant	Lines positive by PCR	Transformation efficiency *
A0	19	41	2.2 bc	-	-	
AI	27	139	5.3 a	20	-	
AII	38	141	3.8 b	28	9	6.4
B0	5	7	1.5 c	-	-	
BI	15	26	1.7 c	-	-	
BII	14	31	2.4 bc	-	-	

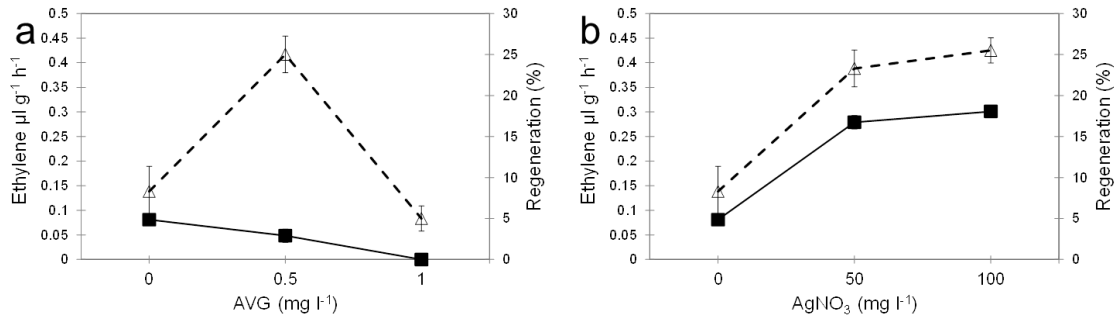
502 *Transformation efficiency was calculated as the percentage of the number of lines positive by PCR
 503 analysis out of the total number of regenerated shoots.
 504

505 Figure 1. Daily rate of ethylene accumulation (■) in the flasks and regeneration efficiency (Δ) in leaf
 506 explants of cultivar Stella at different AVG (a) and AgNO₃ (b) concentrations. The ethylene
 507 accumulation was detected every 24 hours for period of 5 days. Six replications were considered per
 508 treatment, and the experiment was repeated three times. Symbols represent the average and bars ± se.

509
 510 Figure 2. a) Identification of lethal dose in regenerated shoots through kanamycin dosage curve analyses.
 511 b) Bacterial growth on LB media enriched with different amount of AgNO₃.

512
 513 Figure 3. Synergistic effect of AVG, AgNO₃ and *Agrobacterium* co-cultivation on regeneration
 514 efficiency of leaf explants of cv Stella. A0 = leaf explants co-cultivated with bacteria, B0 = control leaf
 515 explants, AI = leaf explants co-cultivated and treated with AVG, AII = leaf explant co-cultivated and
 516 treated with AgNO₃, BI = leaf explants not co-cultivated and treated with AVG, BII = leaf explants not
 517 co-cultivated and treated with AgNO₃. Histograms represent the average and bars indicate ± se, different
 518 letters indicate statistically differences.
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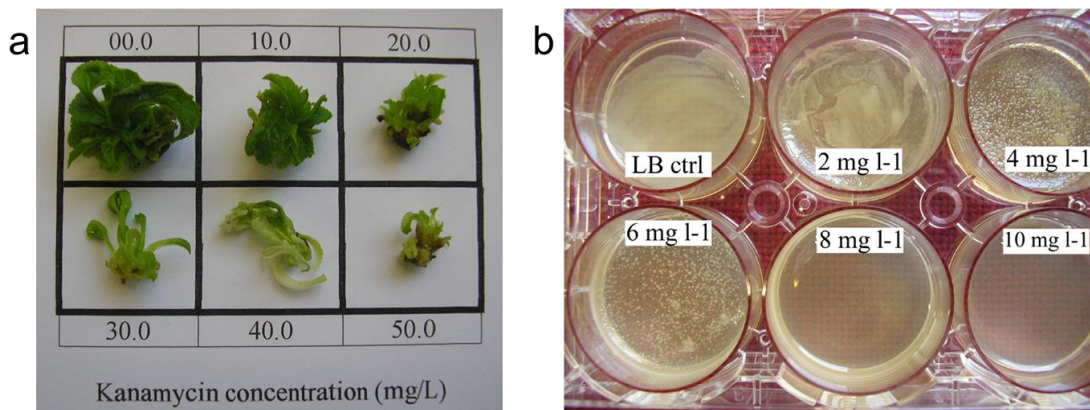
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522 Figure 1

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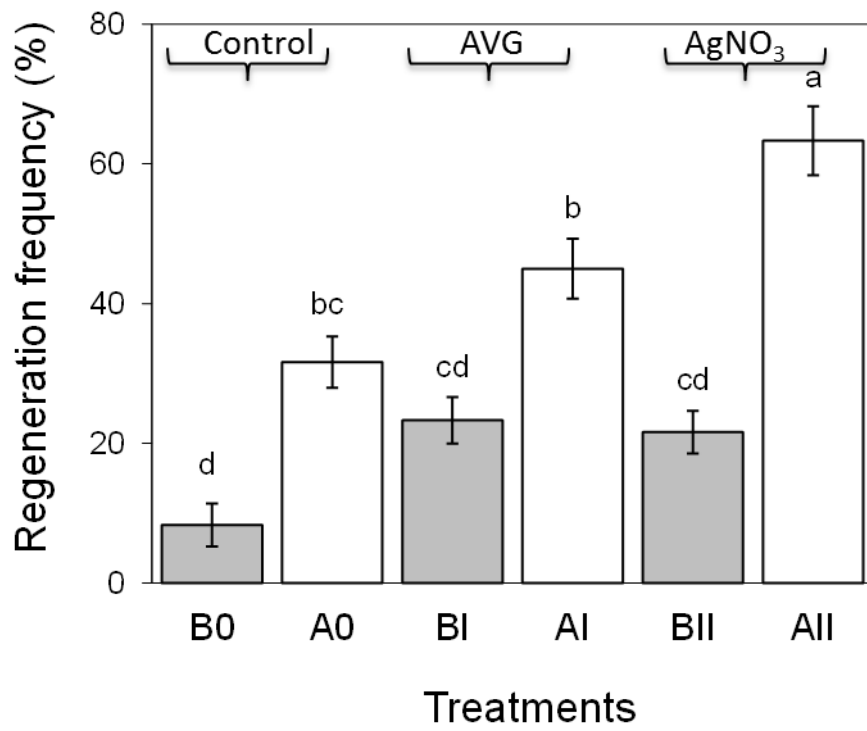
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527 Figure 2

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530 Figure3

To The Editor
Plant Cell, Tissue and Organ Culture (PCTOC)

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05 May 2014

Dear Sir:

I am submitting the manuscript

“Ethylene Inhibitor silver nitrate enhances regeneration and genetic
transformation of *Prunus avium* (L.) cv Stella” by Sgamma *et al.*

for consideration as research article to publish in Plant Cell, Tissue and Organ
Culture.

I can confirm that this work has not been submitted for consideration for
publication elsewhere either in full or in part and solely the corresponding
author will handle the communication with the editorial office. Besides, all
authors confirm that the data acquisition was not in legal conflict with the
authorities where the work was carried out.

We selected The *Plant Cell, Tissue and Organ Culture (PCTOC)* rather than
other journals because of The *PCTOC*'s capacity for disseminating cutting
edge research, in this specific research sector.

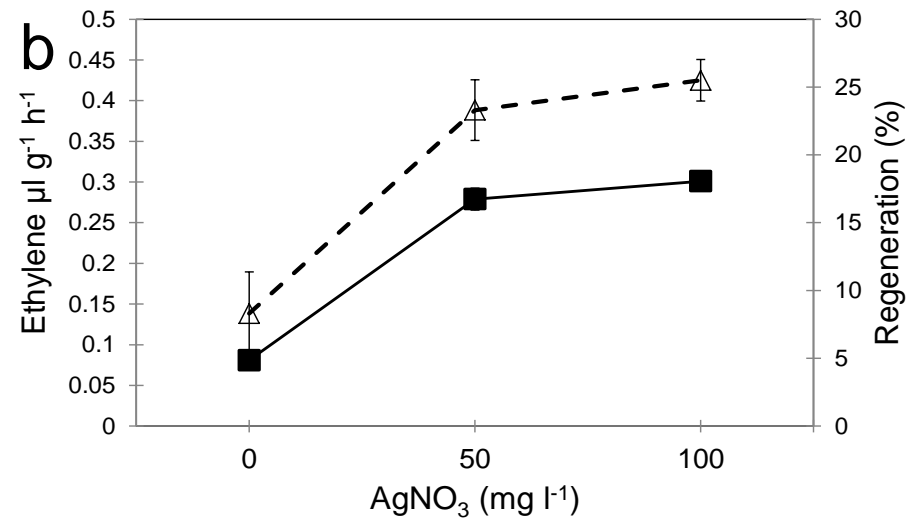
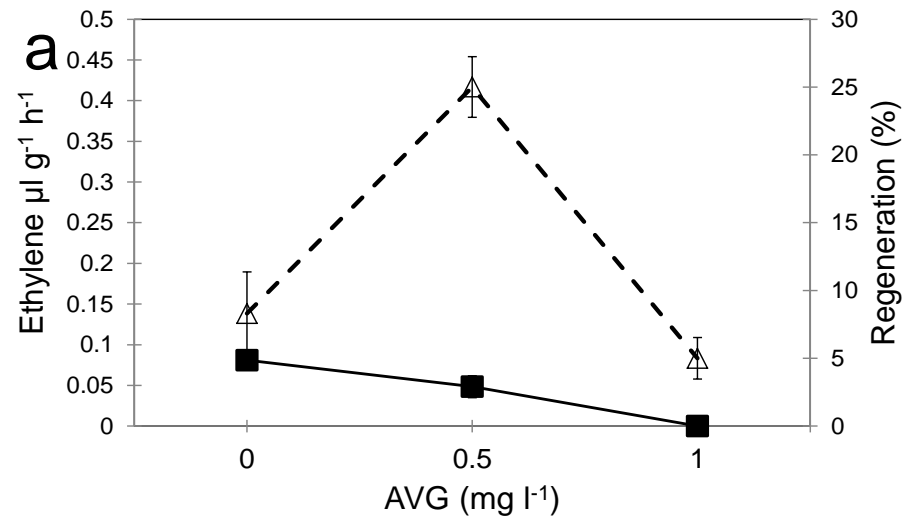
Our article will engage a broad spectrum of interest from plant
biotechnologists as it enlarges the possibility to cherry *in vitro* regeneration
and alien gene transfer, by interfering with the role of ethylene, in a very
established *in vitro* model system for recalcitrant woody plants, leading to a
wide range of applications. Phytochrome allelic enrichment could even be a
biological model that aids in the discovery of signal transduction in plant-
environment interactions.

Please address all communications regarding the manuscript to myself using
the above contact information.

I look forward to receiving your decision.

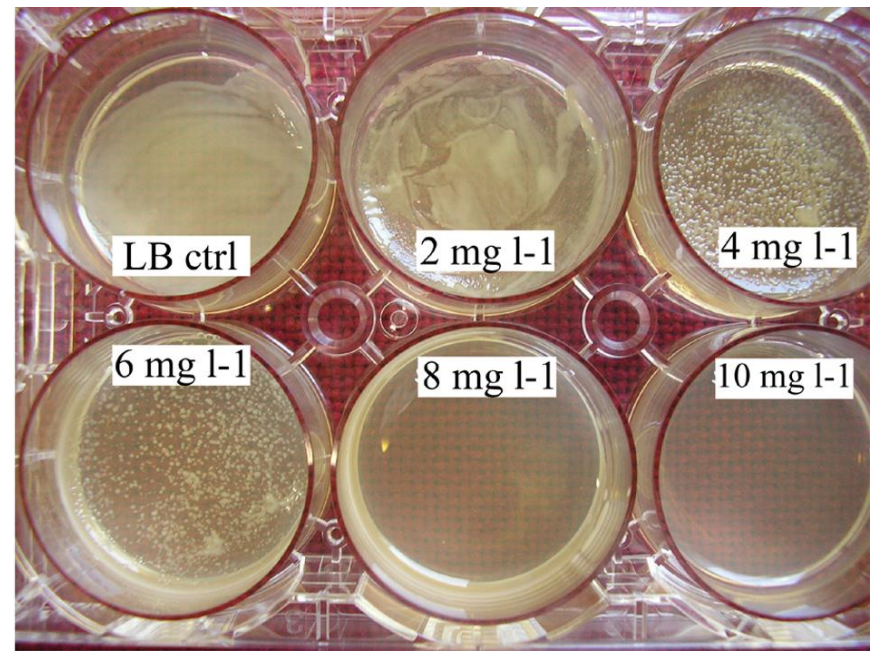
Yours sincerely

Professor Rosario Muleo



a

Kanamycin concentration (mg/L)

b

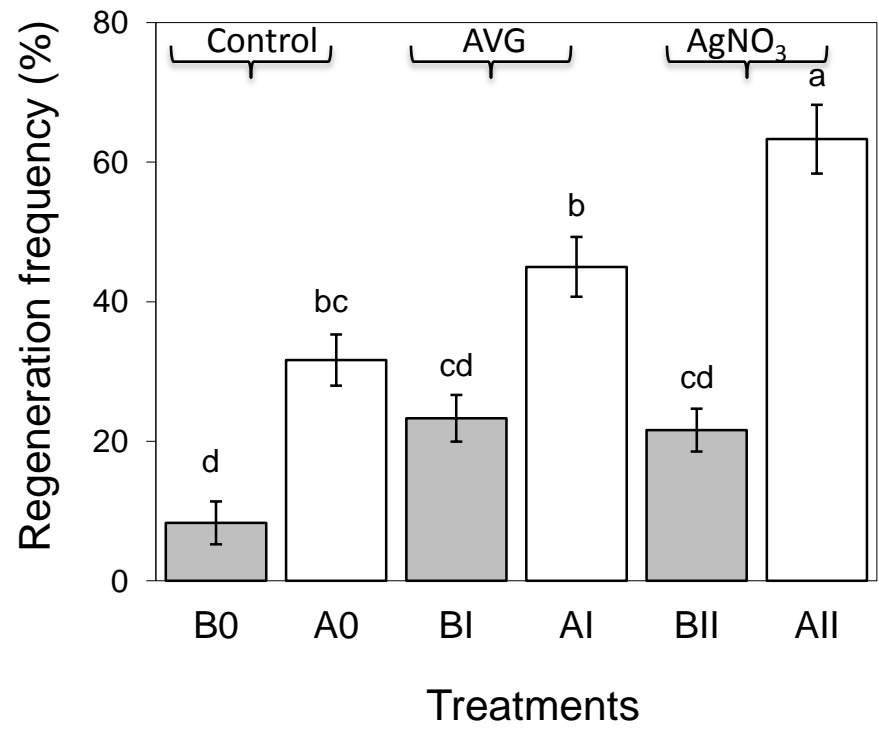


Table 1: Effect of AVG (I) and AgNO₃ (II) applied after co-cultivation with *Agrobacterium* (A) or alone (B) or on the regeneration frequency (number of regenerated shoots per regenerating explant) and transformation efficiency, as recorded in the 3rd trial. Sixty explants were used for each treatment. Means within a column followed by the same letter are not significantly different by Tukey's test (P<0.05).

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*Transformation efficiency was calculated as the percentage of the number of lines positive by PCR analysis out of the total number of regenerated shoots.

1 **Ethylene Inhibitor silver nitrate enhances regeneration and**
2 **genetic transformation of *Prunus avium* (L.) cv Stella.**

3

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10 Key Message: High regeneration and transformation efficiency in sweet cherry.

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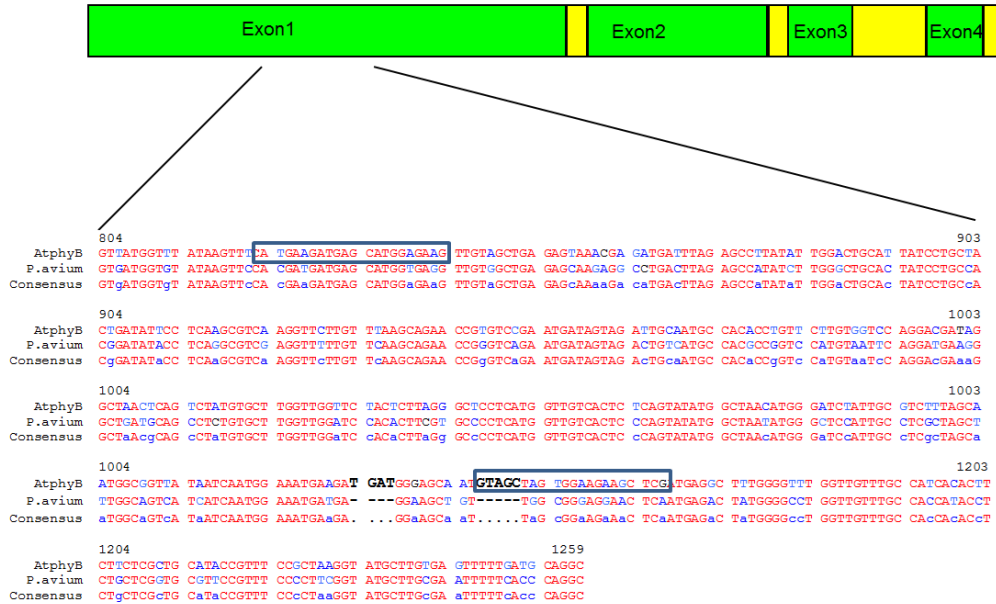
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31 Online Resource 1: Graphic representation of *Arabidopsis phyB* gene (AY466496) and alignment with *P.*
32 *avium phytochrome B*. In blue are shown the polymorphisms and in black the mismatches between the two
33 sequences. In the boxes are shown the primers selected for specific amplification of *AtphyB*.

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Online Resource 2: Primer sequence and fragment size of the analysed genes.

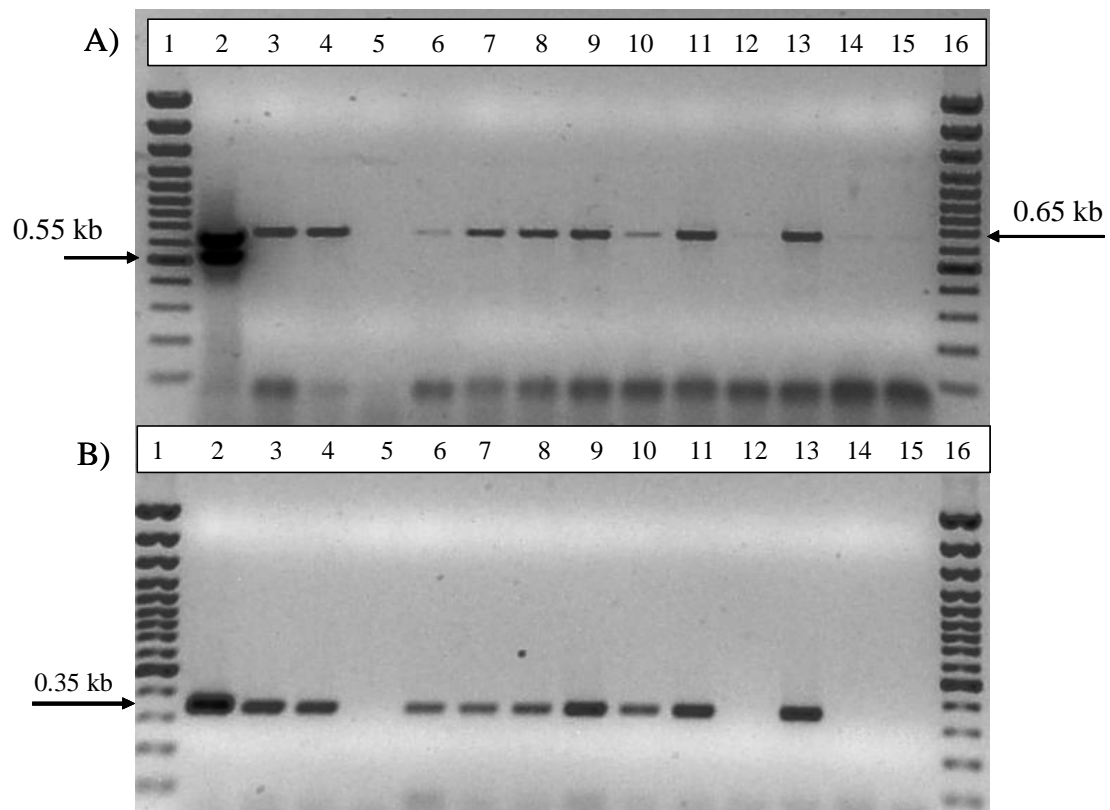
gene	Expected fragment size (bp)	Primer sequence	Annealing temperatures
<i>AtphyB</i>	345	cat gaa gat gag cat gga gaa g (forward) cga gct tct tcc act agc tac (reverse)	59° C
<i>nptII</i>	650	atg gat tgc acg cag gtt etc (forward) cca acg cta tgt cct gat agc (reverse)	58° C
<i>trpR</i>	550	act cct ctt acg gcc ata tcg (forward) atg cgc acc ctg ata acg agc (reverse)	55° C

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44 Online Resource 3. Molecular confirmation of the transgene insertion and no contamination of bacteria.
 45 (A) PCR amplification for *nptII* (0.65 kb) and *trpR* (0.55 kb) fragments, and for (B) *A. thaliana phyB*
 46 (0.35 kb) fragment using genomic DNA from 11 individual cherry lines putative transformed with the
 47 *phyB-nptII* construct pROK. Lanes 1 and 16 Ladder plus (MBI Fermentas); lane 2, positive control *nptII*
 48 amplified plasmid plus *trpR* amplified bacterial DNA, (A), and positive *phyB* amplified plasmid (B);
 49 lanes 3-13, individual putative transgenic cv 'Stella' lines S1, S3, S4, S5, S9 S11, S15, S16, S23, S25,
 50 S27; lane 14, negative control (WT); lane 15 negative control (water).
 51