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Studies on genetic transformation of olive (*Olea europaea* L.) somatic embryos: I. Evaluation of different aminoglycoside antibiotics for *nptII* selection; II. Transient transformation via particle bombardment

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Abstract As a first step to the establishment of a genetic transformation protocol for olive somatic embryos obtained from the seeds of cv. 'Picual', the efficiencies of different aminoglycoside antibiotics as selective agents to be used with the *nptII* marker gene, and the particle bombardment technique for transient transformation have been evaluated. Among the three antibiotics tested, paromomycin and kanamycin showed a similar inhibitory effect and, at 200 mg l^{-1}, both of them impaired callus growth after 8 weeks of culture. However, when isolated embryos were cultured in the presence of these antibiotics, a 20% of the embryos still remained viable at 400 mg l^{-1}. Neomycin was discarded as a selective agent since it showed only a moderate toxic effect. Contrary to solid medium, when olive callus was cultured in liquid medium supplemented with different paromomycin concentrations for 3 weeks, the callus growth was impaired at the lowest antibiotic concentration, 3 mg l^{-1}. Best conditions for transient transformation of olive callus using PDS-1000/He system were a 6 cm target distance and a 900 psi bombardment pressure. pCGUΔ1 plasmid, containing the *gus* gene under the control of sunflower ubiquitin promoter yielded a significantly higher number of *gus* expression areas per bombarded explant than pGUSINT or pJGUS5 plasmids, where the *gus* gene is driven by *CaMV35S* promoter or *CaMV35S* with enhancer, respectively. Almost 45% of bombarded explants showed *gus* expression 12 weeks after bombardment.

Abbreviations
DKW   Driver and Kuniyuki medium
ECO   Olive cyclic embryogenesis medium
MS   Murashige and Skoog medium
OEC   Olive embryogenic calli
OMe   Olive medium

Introduction

The olive (*Olea europaea* L.) is an economically important crop typically cultivated in the Mediterranean basin, although its distribution area is currently being extended worldwide e.g. Australia, China and South America (Rugini and Gutiérrez-Pesce 2006). Several breeding programs are currently under way to improve the olive cultivars; main objectives of these projects are the increase in production, the improvement of oil content and quality, the modification of vegetative growth behaviour and the resistance to several biotic and abiotic stresses. However, traditional olive breeding is hampered by several factors such as the long juvenile phase of the seed progeny, the low level of fruit set and the slow and erratic seed germination (Acebedo et al. 1997). In recent years, biotechnological techniques are being implemented to help breeding and, for example, the first linkage map of the olive genome has been generated (Rosa et al. 2003). Cloning of several genes related to important traits has also been reported (Rugini and Baldoni 2005). Nevertheless, the development of genetic transformation techniques in olive is still a very difficult task,

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mainly due to the recalcitrant nature of olive tissues to regenerate in vitro. 'Picual' is the most popular olive cultivar in Spain, hence, as a first step to its genetic improvement through biotechnological methods, we have recently developed an efficient regeneration system of juvenile material via somatic embryogenesis using excised radicles from mature zygotic embryos as explants (Pérez-Barranco et al. 2007).

In addition to a DNA-delivery system, a successful plant transformation requires an efficient selection procedure to allow the regeneration of transgenic cells and to inhibit the proliferation of non-transformed sectors. The characterisation of this transformation parameter in olive is still deficient. Rugini et al. (2000) and Mencuccini et al. (1999) reported the use of neomycin phosphotransferase II (NPTII), as a selective marker, and the selective agent, kanamycin to get transgenic olive plants. The *nptII* gene encodes a protein which is capable of inactivating aminoglycoside antibiotics through phosphorylation (Miki and McHugh 2004). Although kanamycin is the most commonly used, the other aminoglycoside antibiotics, such as paromomycin, neomycin or geneticin, with different biological activity can be employed to select transgenic plants. Furthermore, in several species, it has been shown that the choice of the appropriate antibiotic has a significant effect on the selection and the transformation efficiencies (Norelli and Aldwinckle 1993; Petri et al. 2005; Shin et al. 2007). When using the *nptII* marker gene, it is therefore advisable to test the efficiency of different aminoglycoside antibiotics for each species and the explant prior to develop a transformation protocol.

Few attempts on olive genetic transformation have been reported so far. The *Agrobacterium*-mediated transformation of cultivar 'Canino' with *rolABC* genes from *A. rhizogenes* to modify vegetative growth and an osmotin gene for increasing resistance to biotic stress has been reported (Rugini et al. 2000; Rugini and Gutiérrez-Pesce 2006; D'Angeli and Altamura 2007). Mencuccini et al. (1999) also used *A. tumefaciens* to introduce *rolABC* genes in cv. 'Dolce Agogia', using petioles as initial explants. Although transgenic callus was obtained, regenerated shoots did not survive in a medium supplemented with 100 mg l^{-1} kanamycin. In species difficult to transform via *Agrobacterium*, particle bombardment has proven to be an adequate transformation system. The development of a transformation protocol via biolistics requires the optimisation of several parameters, e.g. bombardment pressure, target distance, microprojectile nature and size, that can vary substantially depending on the explant used (Sanford et al. 1993). In olive, Lambardi et al. (1999) tested different devices and bombardment conditions for the transient transformation of somatic embryos of cv. 'Canino'. More recently, Pérez-Barranco et al. (2007) obtained transient

expression of the *gus* gene in olive embryogenic cultures using the PDS-1000/He system.

In this investigation, we have evaluated several aspects of the genetic transformation of olive. The main goals of this study were: First, to determine the efficiency of different aminoglycoside antibiotics as selective agents. Second, to characterise the best conditions for transient transformation of embryogenic callus via particle bombardment.

Materials and methods

Plant material and culture conditions

Olive embryogenic cultures derived from mature seeds of cv. 'Picual' were induced according to Orinos and Mitrakos (1991). Briefly, radicle segments of mature seeds were cultured in OMc medium (Cañas and Benbadis 1988) supplemented with 25 μM IBA and 2.5 μM 2iP for 3 weeks, and later transferred to OMc medium without hormonal supplement. Embryogenic callus developing at the surface of the explants was isolated and cultured in olive cyclic embryogenesis medium (ECO) as described by Pérez-Barranco et al. (2007). This basal proliferation medium contained the macronutrients of OMe medium (Cañas and Benbadis 1988), ½ OM vitamins, ¼ MS microelements (Murashige and Skoog 1962), 50 mg l^{-1} myo-inositol, 20 g l^{-1} sucrose, 550 mg l^{-1} glutamine, as well as the following supplements, 0.25 μM IBA, 0.44 μM BAP, 0.5 μM 2iP, 200 mg l^{-1} cefotaxime and 1 g l^{-1} caseine hydrolisate, according to Rugini and Caricato (1995); the medium was solidified with phytagel 3 g l^{-1}. Olive embryogenic calli (OEC) were cultured in the dark at 25 ± 2°C, and subcultured onto a fresh medium every 4 weeks.

Somatic embryos differentiated following transfer of callus to basal ECO medium, e.g. without either growth regulators or cefotaxime, and supplemented with 1 g l^{-1} activated charcoal. Germination took place in a modified solid MS medium with 1/3 macroelements and 10 g l^{-1} sucrose (Clavero-Ramírez and Pliego-Alfaro 1990).

All culture media were adjusted to pH 5.74 with NaOH or HCl before adding the solidifying agent. Afterwards, media were autoclaved at 121°C and 0.1 MPa for 20 min and distributed in Petri dishes or 25 × 150 mm test tubes covered with kaputs (Bellco Glass Inc.). Cefotaxime was filter-sterilised and added to the cooled medium after autoclaving.

Selection experiments

The effect of the antibiotics was tested in three different types of explants, e.g. embryogenic masses, isolated

embryos and isolated nodal segments, 1.5 cm in length with axillary buds, obtained from germinated somatic embryos.

The effect of the aminoglycoside antibiotics—paromomycin, neomycin and kanamycin—at 0, 50, 100 and 200 mg l^{-1} on the growth of OEC was tested. Embryogenic masses (0.1 g) were cultured in 25 × 150-mm test tubes, containing 15–20 ml of ECO medium supplemented with one of the three antibiotics. OEC were weighted after 4 weeks of culture and subcultured onto a fresh medium. Data were taken during the three subcultures. Ten tubes per antibiotic and concentration were used.

The effect of kanamycin and paromomycin on the growth of the isolated embryos was also tested. For this purpose, globular somatic embryos (1–3 mm diameter) were cultured on Petri dishes containing ECO medium supplemented with paromomycin or kanamycin at 0, 50, 100, 200 and 400 mg l^{-1}. Embryos were recultured onto a fresh medium every 2 weeks and the percentage of dead explants was recorded after four subcultures. Twenty-five embryos per treatment were employed.

To test the efficiency of selection in liquid media, 0.4 g of friable embryogenic calli were grown on 250-ml culture flasks containing 40 ml of liquid ECO medium supplemented with paromomycin at 0, 3, 12.5 and 25 mg l^{-1}. Suspensions were incubated in semi-darkness in an orbital shaker at 120 rpm for 3 weeks, and later recultured in solid medium without antibiotics. Five flasks per treatment were employed.

Finally, the development of axillary buds in the presence of paromomycin at 0, 50 100 and 200 mg l^{-1} was assessed. Nodal segments derived from the germinated embryos were cultured in DKW medium (Revilla et al. 1996) solidified with 6 g l^{-1} agar in the presence of antibiotics for 6 weeks and then, the length, the number of leaves and the general aspect of developing shoots, as well as the size of the basal callus were recorded. Twenty nodal sections per treatment were employed. These cultures were incubated under a 16 h photoperiod of 35 µmol m^{-2} s^{-1} at 25 ± 2°C.

In all experiments, antibiotics were filter-sterilised and added to the cooled media after autoclaving. Paromomycin stock solution was adjusted to pH 9 to avoid precipitation in media gelled with phytagel (Kapaun and Cheng 1999). All selection experiments were conducted twice.

Microprojectile bombardment

Microprojectile delivery of DNA in somatic embryogenic cultures was carried out using the PDS-1000/He system from Bio-Rad. Plasmid pCGUΔ1 (Binet et al. 1991) harbouring the gus gene under the control of the sunflower ubiquitin promoter and the nptII for kanamycin resistance was coated into tungsten m-20 microprojectiles as described by Sanford et al. (1993). Target tissue for bombardment was 0.5 g of embryogenic cultures, selecting only the proembryogenic masses of small size (1–3 mm diameter). Explants were placed in a circumference of ∼2 cm diameter, at the centre of a Petri dish containing ECO medium. These explants were bombarded with 6 µl of the DNA-coated microprojectile suspension using the following standard conditions: a 680 mm Hg vacuum pressure, a gap distance from the rupture disc to the macrocarrier of 0.6 cm and a macrocarrier flight distance of 1.1 cm. After bombardment, the plates were incubated in the dark for 48 h prior to assess the GUS activity by histological assay (Jefferson 1987). The efficiency of the transient transformation was estimated as the number of foci showing GUS activity per bombarded callus.

Several bombardment parameters were evaluated. Initially, the effect of target distance, 6 and 9 cm, and bombardment pressure, rupture discs of 600, 900, 1,100 and 1,500 psi, on the transient transformation of OEC was determined. Afterwards, the efficiency of three different plasmids containing the gus gene driven by different promoters, pGUSINT, which included the CaMV35S promoter (Vancanneyt et al. 1990), pJGUS5, with the 35S promoter with an enhancer (Day et al. 1991) and pCGUΔ1 plasmid, was compared. In these experiments, tungsten m-20 particles and a standard DNA concentration for microprojectile coating of 1 µg/µl were used. In a different experiment, the nature of microprojectile was evaluated; e.g. embryogenic calli were bombarded either with tungsten m-20 particles (average size 1.3 µm), gold particles of 1 µm mean diameter or gold particles of 1.6 µm diameter. In the three cases, microcarriers followed the same protocol for sterilisation and they were coated with the pCGUΔ1 plasmid at 1 µg/µl concentration. In a different experiment, the effect of DNA concentration on the expression of gus gene was tested. Thus, microprojectiles, tungsten m-20 particles, were coated with pCGUΔ1 plasmid at 1, 2 or 3 µg/µl delivering 0.6, 1.2 or 1.8 µg of DNA per shot, respectively. Finally, the effect of preculture of explants for 48 h prior to bombardment in ECO medium supplemented with mannitol at 0.2, 0.4 or 0.6 M was analysed. Three to five bombardments per treatment were performed and all experiments were conducted twice.

Following optimisation of the conditions for biolistic transformation of OEC, several attempts were performed to get a stable transgenic line. Thus, the bombarded calli were subjected to selection in ECO medium supplemented with 200 mg l^{-1} paromomycin, delaying the selection phase 1, 10 or 20 days after bombardment. Gus expression was analysed after 8 weeks of selection. In a different experiment, the bombarded calli were cultured in liquid medium containing paromomycin at 3, 6 or 12 mg l^{-1} during

1 week, and later on, recultured in solid medium without antibiotics for three subcultures.

Statistical analysis

Data related to biolistic experiments were subjected to analysis of variance using the SPSS software. Levene′s test for homogeneity of variances was performed prior to ANOVA and the Tukey test at $P = 0.05$ was used to determine significant differences among means. Percentages of necrotic embryos after antibiotic selection were analysed by chi-square test at $P = 0.05$.

Results

Effect of aminoglycoside antibiotics on callus growth

In the first experiment, the effect of the addition of the aminoglycoside antibiotics—neomycin, paromomycin and kanamycin—at 0, 50, 100 and 200 mg l^{-1} on the growth of OEC was tested. Explants (0.1 g of OEC) were subcultured onto a fresh medium at 4 week intervals and data were taken during the three subcultures. In general, the response of OEC to paromomycin and to kanamycin was similar (Fig. 1). Callus growth was not significantly inhibited during the first subculture even at the highest antibiotic concentration, with the exception of paromomycin 200 mg l^{-1} that yielded a strong inhibitory effect. Afterwards, the subculture of explants into a fresh medium increased the sensitivity of OEC to the antibiotics, with callus growth being inhibited at a lower concentration (100 mg l^{-1}). Contrary to these antibiotics, neomycin did not significantly affect the growth of OEC at any concentration during the three subcultures (Fig. 1).

The effect of paromomycin and kanamycin on the proliferation and viability of isolated somatic embryos was also tested (Fig. 2). No statistically significant differences on the percentage of necrotic explants between kanamycin and paromomycin were found at any of the concentrations tested. Kanamycin significantly decreased somatic embryo viability at concentrations higher than 100 mg l^{-1}, with a percentage of necrotic embryos (after 60 days of culture) close to 32% at 100 mg l^{-1}. In the case of paromomycin, a similar percentage of dead somatic embryos was achieved at higher concentrations (200 mg l^{-1}) than kanamycin. At the highest concentration (400 mg l^{-1}), both antibiotics yielded a similar effect, and more than 80% of somatic embryos were dead.

OEC were cultured in liquid medium supplemented with increasing concentrations of paromomycin and the callus weight was recorded weekly (Fig. 3). In the absence of antibiotics, olive callus proliferated exponentially, reaching
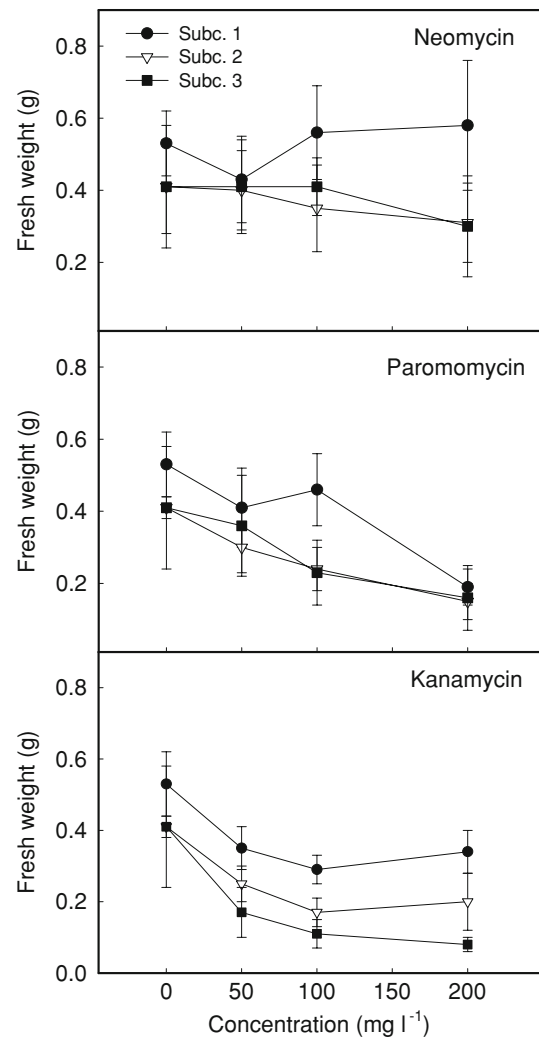

Fig. 1 Effect of different aminoglycoside antibiotics on the growth of olive embryogenic calli. Explants (0.1 g) were subcultured every 4 weeks and data were taken during three subcultures. Mean ± SD of 10 replicates per treatment

a growth rate of 0.043 g day^{-1} after 3 weeks. The addition of paromomycin to the liquid medium totally impaired the growth of OEC even at the lowest concentration, 3 mg l^{-1}. These calli did not recover when transferred to solid medium without antibiotic.

Effect of paromomycin on the growth of axillary buds

To test the efficiency of paromomycin as a selective agent on the micropropagation phase, nodal segments from germinated embryos were cultured in DKW medium supplemented with paromomycin at 0, 50, 100 or 200 mg l^{-1}. In the control treatment, two shoots of different length developed per each nodal segment, with mean length values of 1.38 ± 0.86 and 0.72 ± 0.59 cm (mean ± SD) for the first and the second shoots emerged, respectively. Additionally,
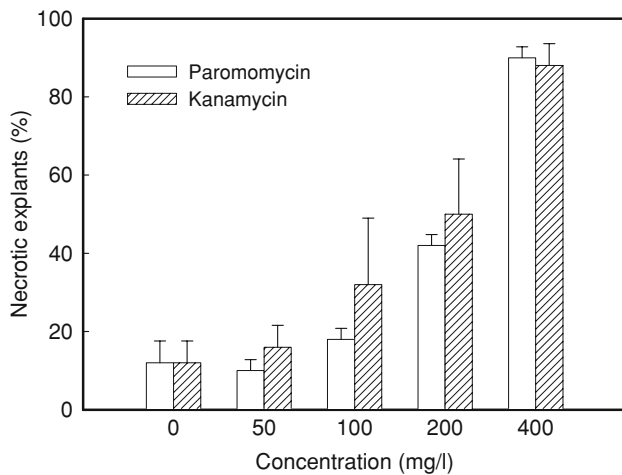
Fig. 2 Percentage of necrotic explants after 60 days of culture on basal proliferation medium supplemented with paromomycin or kanamycin at different concentrations. Data correspond to mean ± SD
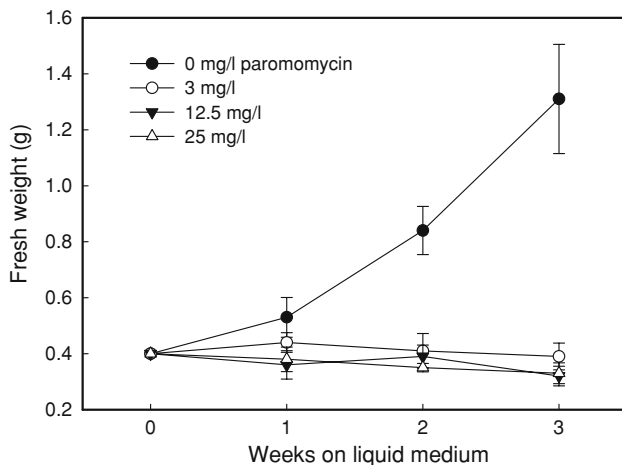

Fig. 3 Effect of paromomycin concentration on the growth of olive embryogenic callus cultured in liquid medium for 3 weeks. Data correspond to mean ± SD

most explants produced a large amount of green callus at the base. The inclusion of paromomycin in the medium inhibited the development of buds at any of the concentrations tested. Most paromomycin treated axillary buds appeared brown after the 6 weeks treatment, in contrast to the green colour of the control buds. Paromomycin also inhibited the production of callus at the base of explants.

Biolistic transformation of OEC

Preliminary experiments showed that plasmid pCGUΔ1 containing the gus gene under the control of sunflower ubiquitin promoter was more efficient for transient expression of gus gene in 'Picual' embryogenic callus than plasmids harbouring the CaMV35S promoter. Thus, this

plasmid was used to optimise the parameters for biolistic transformation of OEC. In the first experiment, explants, 0.5 g of OEC, were bombarded at different target distances (6 and 9 cm) and helium pressures (600, 900, 1100 and 1500 psi), and the number of gus expressing foci per explant was recorded 2 days after bombardment. The results obtained are shown in Fig. 4. In general, a higher number of blue spots per bombardment was obtained at 6 cm target distance. Regarding helium pressure, best results were obtained with 900 psi, followed by 600 and 1100 psi. The lowest number of blue spots was obtained at 1500 psi, independently of the target distance used. The statistical analysis of the data by two-way ANOVA at the 5% level indicated a significant effect of helium pressure ($P = 0.008$), whereas neither the target distance ($P = 0.067$) nor the interaction between both variables ($P = 0.663$) were statistically significant. A pressure of 900 psi and a 6 cm target distance were chosen as the best conditions for subsequent experiments.

Several factors that could influence the efficiency of transient transformation of OEC were evaluated. Plasmids containing the 35S, pGUSINT, or the 35S with enhancer, pJGUS5, promoters driving the gus gene yielded a number of blue spots per shot 10 to 5 fold lower than that obtained with the pCGUΔ1 plasmid, harbouring the sunflower ubiquitin promoter (Table 1). In the above described experiments, a standard plasmid DNA concentration of 1 μg/μl was used to coat tungsten m-20 microprojectiles, delivering about 0.6 μg per shot. The increase in DNA concentration to deliver 1.2 or 1.8 μg per bombardment slightly increased the number of blue spot per callus, although the differences with the standard concentration were not statistically significant (Table 1). Regarding the type of microprojectiles, the best results were obtained with
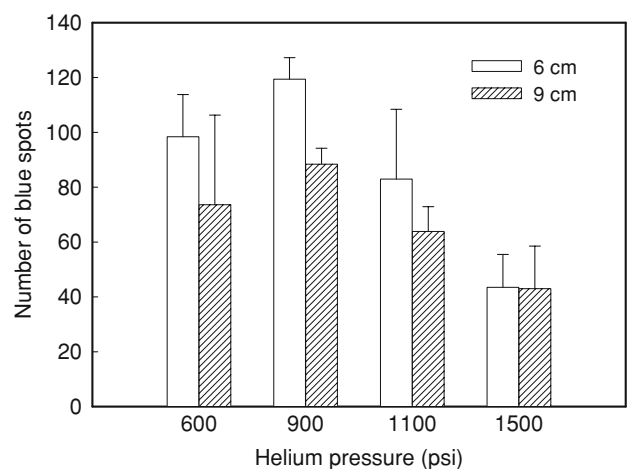

Fig. 4 Effect of target distance and helium pressure on the transient expression of gus gene in olive embryogenic callus. The number of gus expressing foci was measured 24 h after the bombardment. Data correspond to mean ± SD of two independent experiments

Table 1 Effect of plasmid type, DNA per shot and type of micro-projectile on the transient expression of the *gus* gene in olive embryogenic callus

Treatment	Number of blue spots per bombardment
Plasmid	
pCGUΔ1	134a
pGUSINT	12.7b
pJGUS5	27.2b
DNA per shot	
0.6 µg	98.5a
1.2 µg	126.5a
1.8 µg	109.2a
Type of particles	
Tungsten m-20	98ab
Gold particles (1 µm diameter)	124.5a
Gold particles (1.6 µm diameter)	30.5b

Within each parameter evaluated, means with different letter indicate significant differences by Tukey test at $P = 0.05$
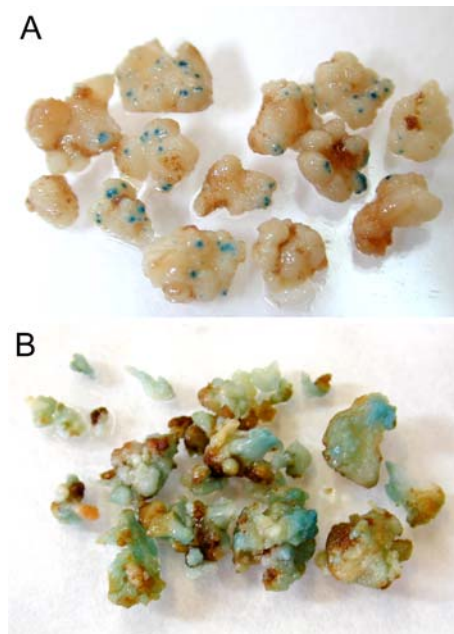

Fig. 5 Expression of the *gus* gene in olive embryogenic callus bombarded with the pCGUΔ1 plasmid. **a** GUS assay performed 2 days after bombardment. **b** GUS assay performed 8 weeks after bombardment. In this case, callus was selected on 200 mg l^{-1} paromomycin, delaying selection 10 days after bombardment

1 µm diameter gold particles, but no significant differences with tungsten m-20 particles were observed (Table 1). Interestingly, the gold particles of larger mean diameter (1.6 µm) yielded a number of blue spots significantly lower than that obtained with the smaller gold microprojectiles. Finally, the preculture of OEC on proliferation media supplemented with different concentrations of mannitol during 48 h, prior to bombardment, did not increase transient transformation (data not shown).

In a first experiment carried out to get a stable transgenic line, a 72.7% of embryogenic calli showed *gus* positive sections after 8 weeks of selection on 200 mg l^{-1} paromomycin. The percentage of *gus* positive explants was slightly higher (77.7%) when selection was delayed 10 days after bombardment, but decreased to 60% when selection was delayed 20 days. These *gus* expression areas corresponded mainly to diffuse sections although single spots were also observed (Fig. 5). However, no calli showed a homogeneous expression of the *gus* gene, and large non-transgenic sectors were detected. In a second experiment, bombarded explants were cultured in liquid medium with different paromomycin concentrations during 1 week and later recultured in solid proliferation medium without antibiotics. After 12 weeks of culture, 43% of bombarded calli showed *gus* expression, but even in this case, explants contained portions of non-transgenic sectors.

Discussion

Neomycin phosphotransferase II is the selectable marker most broadly employed in genetic transformation of plants (Miki and McHugh 2004). This gene inactivates the aminoglycoside antibiotics such as kanamycin, paromomycin, neomycin and the gentamicin derivative geneticin by phosphorylation. Among these antibiotics, kanamycin is the selective agent most commonly used, normally at concentrations in the range of 50–200 mg l^{-1}. However, some species and/or explants are especially resistant to kanamycin selection (Escandon and Hahne 1991; Wakita et al. 2001; Sánchez et al. 2005). In our case, the olive embryogenic masses showed a high tolerance to aminoglycoside antibiotics when growing in solid medium, and a high antibiotic concentration (200 mg l^{-1}) and at least two subcultures in the selection medium were necessary to impair callus growth. Among the three antibiotics tested, the neomycin showed a little effect on olive callus growth, and therefore was discarded as a selective agent. In other systems, such as sugar beet (Catlin 1990) and apple (Norelli and Aldwinckle 1993), this antibiotic is considered to be more adequate than kanamycin as a selective agent due to its relatively low toxicity. Moreover, at low concentrations, it stimulates shoot regeneration and elongation (Norelli and Aldwinckle 1993). Regarding kanamycin and paromomycin, both the antibiotics produced a similar effect on the growth of olive embryogenic calli. However, our results showed that paromomycin at 200 mg l^{-1} restricted callus growth earlier than kanamycin, although, after several weeks of culture, the negative effect of kanamycin on the viability of somatic embryos seemed to be stronger than that

produced by paromomycin. These traits would make paromomycin a better agent than kanamycin for selection of transgenic olive cells. Similarly, paromomycin induced an earlier toxic effect on *Vitis vinifera* cell suspensions than kanamycin (Wang et al. 2005). Paromomycin has been successfully used as a selective agent in the transformation of sorghum (Howe et al. 2006), sweetpotato (Shin et al. 2007), *Hevea brasiliensis* (Montoro et al. 2003), cassava (González et al. 1998) and apple (Norelli and Aldwinckle 1993). However, in other species, such as the Siberian elm, it was inadequate for selection because this antibiotic did not completely inhibit the callus growth and the shoot regeneration, even though the tissues showed necrosis (Kapaun and Cheng 1999). Interestingly, when olive embryogenic callus was cultured in proliferation medium gelled with agar instead of phytagel, the toxic effect of all the antibiotics tested was significantly enhanced (data not shown). A similar result was obtained by Laine et al. (2000) in the regeneration of flax hypocotyls on kanamycin supplemented media solidified with different gelling agents. Unfortunately, agar can not be used in our olive system because the calli lost their embryogenic capacity when cultured for several weeks in the proliferation media gelled with this agent (Pérez-Barranco et al. 2007). Contrary to solid media, the growth of olive callus is completely inhibited when grown in liquid media for 3 weeks, even at low paromomycin concentrations such as 3 mg l^{-1}. A similar paromomycin level was used in the selection of *Vitis vinifera* cell suspensions by Wang et al. (2005). Altogether, our results indicate that the olive embryogenic masses need an intimate contact with the selective agent in the medium for a successful selection. The alternate use of solid and liquid media supplemented with 200 and 10 mg l^{-1} of paromomycin, respectively, could be therefore the best protocol for olive selection.

Microprojectile bombardment is an efficient technique for stable transformation of species recalcitrant to *Agrobacterium* infection, and also for transient transformation studies. In this investigation, we have analysed some physical and biological factors to get the best conditions for transient transformation of olive embryogenic callus by using the PDS-1000/He system. Among the different variables tested, the promoter driving the *gus* gene was the most critical factor determining the efficiency of transient GUS expression. Sunflower ubiquitin promoter yielded a significantly higher number of *gus* expressing foci per bombardment than *CaMV35S* promoter, even when the last one contains an enhancer which generally increases the strength of this constitutive promoter. Similarly, Lambardi et al. (1999) found that the sunflower ubiquitin promoter yielded a higher number of *gus* expression units than the *CaMV35S* when olive embryos cv. 'Canino' of small size (at the torpedo stage) were bombarded by using the Particle Inflow Gun device. However, embryos at a more advanced developmental stage (cotiledonary stage) expressed more efficiently the *gus* gene when transformed with the CaMV3S promoter. In other systems, such as Indica rice suspension cultures or sorghum immature embryos, the ubiquitin promoter gave also a significantly higher number of transient expression foci than *CaMV35S* (Li et al. 1997; Tadesse et al. 2003). Our results seem to indicate that the constitutive promoter *CaMV35S* is a weak promoter for transgene expression in young olive somatic embryos. Along this line, Tian et al. (2000) found that the *CaMV35S* promoter was more active in an organised tissue of mature alfalfa somatic embryos than in the less-organised tissues of young embryos.

Besides promoter, physical parameters of the biolistic system such as the helium pressure and the size of microparticles also exerted a great influence on the efficiency of transient transformation. Best transformation rates were obtained at 900 psi helium pressure and 6 cm target distance. Lambardi et al. (1999) found that a slightly higher pressure, 1100 psi, was optimal for a transient transformation of 'Canino' somatic embryos, but in that case they used a larger target distance, 12 cm. Regarding the size of microparticles, the use of large particles significantly reduced the number of *gus* expressing foci. According to Sanford et al. (1993), the size of the particles chosen for biolistic transformation should be about one tenth the diameter of the target cell. An observation of the histological analysis of olive somatic embryos performed by Benelli et al. (2001) showed that small particles of about 1 μm comply with this rule.

Using the best parameters for the transient transformation, we performed several experiments to test the efficiency of biolistic on stable transformation of olive embryogenic callus. In all experiments, a significant percentage of bombarded explants showed the sectors expressing *gus* activity after 8–12 weeks of culture. This result likely indicates the stable integration of transgene in these cell clusters, although this conclusion should be confirmed by molecular analysis. Even more, in most cases, these *gus* expressing areas apparently formed as result of transgenic cell proliferation, since they appeared as diffuse blue areas rather than as single spots. Unfortunately, the different selection protocols tested were unsuccessful because the bombarded calli still contained non-transgenic sectors. A longer selection procedure combining liquid and solid medium should be, therefore, necessary to completely eliminate non-transgenic cells. Alternatively, the use of the GFP marker gene could be very useful in the early selection of transgenic cell clusters after biolistic bombardment, as it has been shown in embryogenic callus of sugarcane (Elliott et al. 1999) or walnut (Escobar et al. 2000), transformed via *Agrobacterium* inoculation.

In conclusion, we have shown that the olive embryogenic calli displayed a high tolerance to antibiotic selection when cultured in solid media, although brief periods in liquid medium supplemented with a low antibiotic concentration constrain the growth of calli. Optimal parameters for transient transformation of embryogenic calli have been established. The transformed explants showed GUS activity several weeks after bombarded. These results indicate that the microprojectile bombardment could be a useful transformation system for olive.

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