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Full Length Article

Producing Transgenic Thompson Seedless Grape (*Vitis vinifera*) Plants using *Agrobacterium tumefaciens*

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Abstract

Under osmotic stress plant avoid it by different modification in their metabolisms such as increasing the synthesis of mRNA of delta 1-pyrroline-5-carboxylate synthase (P5CS), where *P5CS* is a target gene for increasing proline production and expect to improve the resistance to abiotic stress. To test this hypothesis, grape (*Vitis vinifera* L.) cv. Thompson Seedless was conducted to develop a protocol for high frequency regeneration and *Agrobacterium* mediated *P5CS* gene transfer. Callus induction was achieved by culture leaf explants on Nitsch and Nitsch (NN) basal medium including 2.0 mg L^{-1} 2,4-di-chloro-phenoxy-acetic-acid + 0.5 mg L^{-1} Thiadiazuron solidified with 2.5 g L^{-1} phytagel. While for *in vitro* proliferation of plant, the calli were cultured on NN medium supplemented with 3.0 mg L^{-1} Zeatin riboside + 0.5 mg L^{-1} Thiadiazuron. *Agrobacterium*-mediated transformation using the strain LB4404 harbouring the binary vector pBI121 with the *P5CS* gene under CaMV 35S promoter and the bar gene as a plant selectable marker was used for transforming grape explants. A *P5CS* specific band (2100 bp) was amplified from DNA extracted only from the transgenic grape plants and 24% for *P5CS* gene was positive for the bar gene. Over expression of the abiotic stress *P5CS* gene was enhanced synthesis of proline to over 6 times higher in transgenic plants compared to controls. The successful transformation of genetically diverse grape cv. Thompson Seedless indicated that the transformation system may have general application to an even wider range of grapes cultivars. © 2016 Friends Science Publishers

Keywords: Abiotic stress; Grape; Genetic transformation; Gene expression; *P5CS* gene; Proline
Abbreviations: 2,4-D 2,4-di-chloro-phenoxy-acetic-acid; *bar* gene Phosphinotricin acetyl transferase (gene); BAP Benzylaminopurine; HSDT Honestly Significant Difference Test; LB Luria broth medium; MCS multiple cloning site; MS Murashige and Skoog's medium; NN Nitsch and Nitsch medium; IBA Indole Butyric Acid; IPTG Isopropyl-beta-D-1-thiogalactopyranoside; TDZ Thiadiazuron (NphenylN1,2,3,Thiadiasol 5-ylurea); OD Optical density; P5CS 1-pyrroline-5-carboxylate synthase; ROS Reactive oxygen species; ZR Zeatin Riboside.

Introduction

The grape (*Vitis vinifera*) is one of the earliest cultivated fruit trees and its cultivation has spread to nearly all climatically suitable regions of the world, especially in the countries of the Mediterranean, Middle East and Arabian Gulf region. There are many challenges that hinder expansion of grape cultivation in these areas including abiotic stresses such as salinity, water deficit and high temperature (Baneh *et al.,* 2013; Karimi and Yusef-Zadeh, 2013), which impact negatively on plant growth and crop productivity (Patade *et al.,* 2014).

Many plants attempt to overcome and reduce the impact of osmotic stress conditions by synthesizing and accumulating specific inorganic and organic compounds such as proline, glycine betaine, mannitol and trehalose which have osmoprotectant properties (Parida and George, 2015; Farooq *et al*., 2015). These osmolytes are not harmful and accumulate without impairment of normal physiological function to the plant cells. Their action in the cell is to enhance molecular stability of proteins and cell membranes during dehydrative stress conditions (Wang *et al.,* 2007; Gupta and Huang, 2014). Proline is one of the most accumulated osmolytes found in plants exposed to salinity and water deficit (Tripathi *et al.,* 2007; Takano *et al*., 2009; Khan *et al.,* 2014) and it performs as a compatible solute and reactive oxygen species (ROS) detoxifier (Szabados and Savoure, 2010; Sadak *et al.,* 2015). The action of proline varies among plant organs, for instance it has been found to help redistribute nitrogen to younger tissue in leaves and it also helps counter energy shortages in flowers (Zhang and Becker, 2015). A plant protecting him against the lethal effects of salinity by over accumulation of proline that may be resulting from salinity inducible uptake of exogenous

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proline confers (Fichman *et al.*, 2014). Several studies have highlighted the possible mechanisms of proline accumulation under stress condition; 1- by restricting entrance of non-metabolisable ions such as sodium, transporting them to vacuoles and or excluding them from the cytosol and thus reducing their toxicity (Verbruggen and Hermans, 2008); 2- acting as a non-enzymatic osmoprotectant which suggests it could replace water at protein surfaces, in protein complexes or in membranes Hasegawa *et al.* (2000); 3- acting as both an osmotic agent and a radical scavenger (Kishor and Sreenivasulu, 2014) and; 4- act to balance cell redox status and to buffer cytosolic pH (Maggio *et al.*, 2002).

In plants, proline is synthesized in two ways, by the glutamate pathway and/or arginine/ornithine pathway (Szabados and Savoure, 2010). Through glutamate proline is generated via D1-pyrroline-5-carboxilate (P5C) by P5C synthetase (P5CS) and P5C reductase (P5CR) (Kesari *et al.*, 2012). It has been estimated that the increased expression of the *P5CS* gene plays a major function in regulating proline synthesis in plants under adverse environmental conditions compared with normal condition (Su *et al.*, 2011). The glutamate pathway is considered to be the major path for proline synthesis under harsh conditions. In several plant species, *Phaseolus vulgaris* (Chen *et al.*, 2009), *Medicago truncatula* (Armengaud *et al.*, 2004), *Glycine max* (Mau *et al.*, 2010), reed canary grass (Cong *et al.*, 2014) and sugar cane (Iskandar *et al.*, 2014), cloning the *P5CS* gene and its over-expression results in increased abiotic stress tolerance but there are no reports in the literature for grapes.

Plant tissue culture has frequently been advocated to help emphasise those physiological and biochemical procedures essential in cells that contribute to changes in abiotic stress resistance (Alhasnawi *et al.*, 2014). Furthermore, the availability of efficient and reliable regeneration systems from meristematic explants through tissue culture is the first prerequisite to develop an effective genetic transformation system for grapes. Only a few studies have reported improvement in the technique of transformation and tissue culture in grapes (Li *et al.*, 2004; Mulwa *et al*., 2007; Rubio *et al.*, 2015), thus a widely applicable and simple transformation and regeneration protocol for grape is still awaited (Carimi *et al.*, 2012).

For this study it was hypothesized that either it is possible to clone abiotic stress *P5CS* gene in grapes or *P5CS* gene is associated with proline accumulation and salt resistance?

Materials and Methods

In Vitro Regeneration of Grape (*Vitis vinifera*) cv. Thompson Seedless

Callus formation: The present study was conducted during the years 2013–2015. The leaves of Thompson Seedless were taken from plantlets generated previously on Murashige and Skoog (1962) M&S medium supplied with 0.3 mg L^{-1} Indole Butyric Acid (IBA) and 3.00 mg L^{-1} Benzyl Adenine (BA). Leaf sections (1.5-2.00 cm) were cultured on Nitsch and Nitsch medium (1969) addition to different concentrations (1.00 – 5.00 mg L^{-1}) of 2, 4-dichlorophenoxyacetic acid (2, 4-D) with 0.5 mg L^{-1} thiadiazuron (TDZ) or 1.0 Kinetin in order to optimize the best callus induction media. 15 plates, each containing 6 explants were used per treatment and set in a completely randomized block design (CRBD). The cultures were incubated at 25±2°C in the dark for four weeks to encourage callus induction. Callus formation percentage was assessed after 4 weeks.

***In vitro* regeneration:** Callus pieces were sub-cultured every four weeks onto NN fresh medium. To evaluate the regeneration ability to produce shoots, healthy callus was cultivated on NN based media with different dosages of TDZ (1.0–4.0 mg L^{-1}) or zeatin riboside (ZR) (0.5–3.0 mg L^{-1}) for four weeks. Using pH meter instrument, pH for media was adjust at 5.8 and cultures were kept in a controlled incubators at 25±2°C, 8/16 h (dark/light) with an irradiance 40 µmol m^{-2} s^{-1}. Ten jars, each containing three calli were used per treatment and coordinated in a CRBD. After four weeks the frequency of shoot regeneration (No. of regenerating calli/No. of plated calli × 100), number of shoots and shoot length were recorded.

Rooting and acclimatization of regenerated plants: For rooting formation, shoots (about 3 cm in length) derived from the regeneration stage were cultured on MS medium including 1.0 mg L^{-1} IBA. Four week later, plantlets were moved from the Jars and rinsed under tap water to clean the roots from phytagel taking care to minimize injury. The plantlets were transplanting in plastic pots filled with a soil mixture of peat and sand (1:1), and covered with black polyethylene bags and incubated under 40 µM m^{-2} s^{-1}/16 h/30±1°C in growth cabinets. The plants were hardened by removing the polyethylene bags gradually over a 4 to7 week period.

Transformation System of Grape (*Vitis vinifera*) cv. Thompson Seedless

Isolation and purification of recombinant plasmid DNA from transformed cells: *Agrobacterium tumefaciens* strain LBA4404, harbouring the binary vector pBI121, containing the *P5CS* gene which encodes Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) and the bar gene (bialophos herbicide resistant gene) as a selectable marker under the control of the cauliflower mosaic virus 35S promoter (P35S) and NOS terminator were used for transforming grape explants (Fig. 1). *Escherichia coli* strain DH5α (Promega Corp.) was used for cloning and manipulation of the plasmid (s) used. Purification of plasmid was carried out with Qiaprep Spin Miniprep Kit (Qiagen, Germany) according to the manufacturer manual.

***A. tumefaciens* culture preparation:** *Agrobactrium* strain LBA 4404 containing the pB1121 with bar and P5CS genes were applied in this research. A single clone of bacteria was grown in 5 mL of LB medium + 25µg mL^{-1} Streptomycin + 50 µg mL^{-1} rifampicin + 50 µg mL^{-1} kanamycin at 28°C with 180 rpm for 12 h, then it was sub-cultured into a 50 mL LB medium and grown at 5 h at 28°C with 200 × *g* for 24–36 h. The growth culture was centrifugation at 5000 g for 5 min, then the supernatant was separated and the pellet was re-suspended in 100 mL of medium and incubated at 28°C for 2 h under 180 rpm before used.

Agrobacterium-mediated transformation system: The explants of grape were co-cultivated with *A. tumefaciens* strain LBA4404 with plasmid pB1121. The treated explants will cultured onto medium MS for 2 days. Subsequently, they were transferred to the selection callus induction medium with 3.0 mg L^{-1} of bialaphos and 300 mg L^{-1} of carbinicillin and incubated for 4 weeks for callus formation, then developed callus were transferred to the regeneration medium after up to three subcultures on the same medium. This was performed through two replicates and the numbers of explants in each replicate were 45 with a total number of 90 explants and all experiments were repeated two times.

Cloning the P5CS gene: DNA extracted from grapes explant was used to amplify the *P5CS* gene using specific primers. Amplified fragment of *P5CS* gene that expected at molecular weight (2.100 kb) were cloned into the PCR cloning vector pGEM®-T Easy Vector System (Promega, USA) according to the manufacturer manual. The ligated reaction (cloning vectors plus inserts) was used to transform *E. coli* bacterial cells (XL-1blue).

Transformation of the competent cells: 50 µL of competent cells was added to the ligation mixture and mixed briefly and left under cooling (4°C) for 30 min, then put in water bath under 35°C for 40 sec following by incubation on ice for 3 min. After that, 500 µL of Luria and Broth medium will be added and incubated with shaker in water bath at 250 rpm under 37°C for one hour. One hundred of transformation mixture was plated using a sterile spreader onto LB medium containing 40 µg mL^{-1} x-galactose, 100 µg mL^{-1} ampicillin and 0.5 mM IPTG (Isopropyl-beta-D-1-thiogalactopyranoside). The Petri dishes were incubated for one day under 37°C.

Screening of transformed cell: Detection of transformed competent cells with pGEM®-T Easy was carried out by blue/white colony screening as the vector has a ligated fragment at its MCS (Multiple Cloning Site). The MCS of Blue script lies within the *Lac*Z gene, which when expressed, in response to the presence of synthetic inducer IPTG and produces the enzyme β–galactosidase. This releases an indigo dye from the chromomeric substrate, X-gal, resulting in the formation of a blue colony. Insertion of a DNA fragment within the MCS would result in the failure of *Lac*Z expression, and the consequent formation of white colony, thus, cells transformed with pGEM®-T Easy derivatives were plated on LB–plates, to select and identify recombinants.

Detection of Transgenic Plants

Herbicide leaf painting assays: In order to evaluate the expression of *bar* gene, non-transformed (control) and putative transformed grape leaves were painted according to Chen *et al.* (2008) with BASTA herbicide (a proprietary herbicide containing bialaphos) at a concentration of 2.0 g L^{-1}. After a week the leaves were either unaffected or turned brown (necrosis) were considered as positive and negative results.

PCR assay: Genomic DNAs was extracted from putative transgenic plants, as described by Dellaporta *et al.* (1983) and analyzed by PCR. Two pairs of specific primers were used to identify transformed plants, the first was designed to amplify 540 bp of the *bar* gene (namely PI, 5'-AAA AGC TTC CAC CAT GAG CCC AGA ACG ACG-3' and P2, 5'-AAG GAT CCT TAG ATC TCG GTG ACG G') the second pair was designed to amplify the P5CS gene (namely P3, 5'-TAC TGA GAC TGT GAA GTA GCC T-3' and P4, 5-' ATG GCA TTG CAG GCT GCC GTT A -3'). The PCR temperature profile was as follows: initial denaturation of DNA at 94°C for 5 min, 35 cycles comprised of 1 min denaturation at 94°C, 1 min annealing at 55°C for *P5CS* gene or 60°C for *bar* gene, 1 min elongation step at 72°C followed by a final extension step at 72°C for 7 min.

Determination of free proline content: Leaf samples derived from putative transgenic and control grape plants under nature conditions was used to determine proline content according to McMannus *et al.* (2000).

Statistical Analysis

In this experiments, 15 Petri dishes each containing 6 leaf sections was used per treatment (90) leaf sections was cultured for one set of experiment for grape (*Vitis vinifera*) cv. Thompson seedless. Nitsch and Nitsch medium without growth hormones will considered as control. All the data were analyzed for significance using ANOVA (analysis of variance $p<0.05$) according to Duncan (1955).

Results

In Vitro Callus Induction and Plant Regeneration

Leaf sections of grape (*Vitis vinifera*) cv. Thompson Seedless were used to attain callus induction *in vitro* (Fig. 2a). In order to optimize the best callus induction media leaf sections (0.5–1.0 cm) were cultured on NN medium supplemented with 1.0- 3.0 mg L^{-1} 2,4-D combined with 0.5 mg L^{-1} TDZ or 1.0 Kinetin (Table 1). The data recorded that the highest leaf section survival (98%) was observed on NN basal medium supplemented with 1 mg L^{-1} 2,4-D, whereas the lowest value (87%) was detected with 2.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} kinetin. The NN basal medium supplemented with 2.0 mg L^{-1} 2, 4-D

Table 1: Responses after 4 weeks of leaf section explants to growth regulators concentrations on callus formation and adventitious shoot regeneration response of grape cv. Thompson Seedless via *in vitro*

Growth regulators (mg L^{-1})	Leaf sections survival (%)	Callus formation (%)	Adventitious shoot regeneration (+/-)
2,4-D 1.0 mg L^{-1}	98±3.65a	83±1.02de	+
2,4-D 2.0 mg L^{-1}	91±2.54c	89±1.77cd	+
2,4-D 3.0 mg L^{-1}	89±1.78cd	72±0.89e	+
2,4-D 1.0 mg L^{-1} + TDZ 0.5 mg L^{-1}	96±2.98ab	85±1.40d	++
2,4-D 2.0 mg L^{-1} + TDZ 0.5 mg L^{-1}	95±2.84b	92±2.62c	+++
2,4-D 2.0 mg L^{-1} + kinetin 1.0 mg L^{-1}	87±1.58d	73±0.91e	-

No regeneration= -, Poor= +, Good= ++, very good= +++
Values are presented by mean ± SE Same letters represent no significant differences between means at $P \leq 0.05$ level determined by Tukey's test
Bold value indicated the highest value obtained for the treatment compared to other treatments
2,4-D = 2,4-di-chloro-phenoxy-acetic-acid, TDZ = Thiadiazuron

Table 2: Responses of callus to adventitious shoot induction after 4 weeks to regulators concentrations of the grape cv. Thompson Seedless

Growth regulators (mg L^{-1})	Shoot regeneration (%)	Number of shoots	Shoot length (cm)
TDZ 2.0 mg L^{-1}	18.6±0.54f	2.62±0.19d	0.56±0.05f
TDZ 3.0 mg L^{-1}	22.4±0.65e	2.53±0.18d	0.75±0.08e
ZR 3.0 mg L^{-1}	33.3±0.82d	4.33±0.25ab	1.12±0.10d
ZR 2.0 mg L^{-1}ZR + TDZ 0.5 mg L^{-1}	62.8±1.48b	4.02±0.22b	1.55±0.11c
ZR 3.0 mg L^{-1}ZR + TDZ 0.5 mg L^{-1}	76.4±2.25a	6.38±0.28a	2.25±0.15a
ZR 2.0 mg L^{-1} + TDZ 1.0 mg L^{-1}	66.3±1.49b	4.32±0.24ab	1.58±0.12c
ZR 2.0 mg L^{-1}ZR + TDZ 2.0 mg L^{-1}	52.8±1.09c	3.55±0.20c	1.85±0.13b

Values are presented by mean ± SE Same letters represent no significant differences between means at $P \leq 0.05$ level determined by Tukey's test
Bold value indicated the highest value obtained for the treatment compared to other treatments
ZR = Zeatin Riboside, TDZ = Thiadiazuron

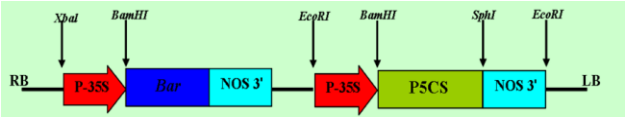

Fig. 1: Schematic representation of the T-DNA of the recombinant binary vector pBI121 carrying the *P5CS* gene driven by CaMV 35S promoter. LB left border, nopaline synthase gene terminator, *P5CS* gene, 35S promoter, nopaline synthase gene terminator, bar gene, 35S promoter, RB Right border

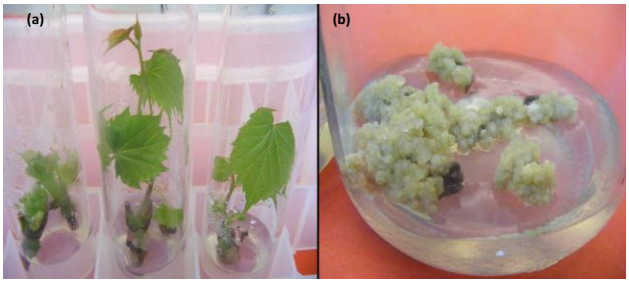

Fig. 2: Shoot formation on MS medium supplemented with 3.0 mg L^{-1} BA and 0.3 mg L^{-1} IBA (a). Callus formation on Nitsch and Nitsch medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} TDZ (b)

and 0.5 mg L^{-1} TDZ was the most effective in callus formation (92%) and shoot formation (+++) (Fig. 2b). To enhance shoot regeneration, callus were cultured on NN basal medium with different concentrations of TDZ or zeatin riboside (Table 2). The best shoot regeneration percentage (76.4%), with a mean length of shoots of 2.25 cm and mean number of shoots of 6.38 was obtained on NN medium plus 3.0 mg L^{-1} zeatin riboside and 0.5 mg L^{-1} TDZ (Fig. 3). MS medium including 1.0 mg L^{-1} IBA was used as an optimized medium for root formation according to Kurmi *et al.* (2011), maintained for 30 days in controlled growth chamber at 25±2°C, 8/16 h (dark/light) with an irradiance 40 µmol m^{-2} s^{-1} supplied with fluorescent lights lamp to generate adventitious roots (Fig. 4a). After four weeks of culture, 98% of the shoots had produced adventitious roots. Nearly all plantlets (96%) moved to greenhouse, showed survival, normal growth and development.

Agrobacterium-mediated Transformation Protocol

Herbicide bialaphos sensitivity: An expected, for control plants an increase in bialaphos concentration was associated with reduced survival of explants percentage. At 0.0, 1.0 and 2.0 mg L^{-1} of bialaphos rates of survival were 95.8, 73.5 and 56.4%, respectively (Fig. 5). The concentration 3.0 mg L^{-1} was considered a lethal concentration of and was subsequently used to select as transformed tissues in. 24 explants out of 100 survived on the bialaphos selection medium and these produced 24 bialaphos-resistant shoots. After 3–4 weeks these developed shoots were sub-cultured to the rooting medium.

Table 3: Mean proline content (µg g^{-1} F W) of transgenic and non-transgenic grape cv. Thompson seedless plants

Plant	Proline content (µg g^{-1} FW)
Transgenic	1960.12 ± 1.7a
Non-transgenic	330.28 ± 0.5b

Two values are presented by mean ± SE, different letters (a and b) represent significant differences at $P \leq 0.05$ using t-test

Fig. 3: *In vitro* regenerated shoots on Nitsch and Nitsch medium supplemented with 3.0 mg L^{-1} ZR + 0.5 mg L^{-1} TDZ

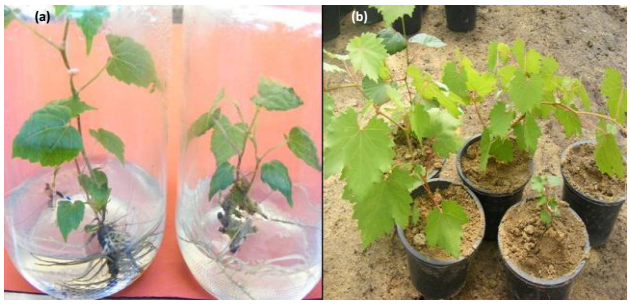

Fig. 4: Well rooted shoots of grape *in vitro* achieved on MS medium supplemented with 1.0 mg L^{-1} IBA (a); Acclimatized and weaned *in vivo* grapes plants derived from *in vitro* regeneration (b)

Molecular Analysis and Gene Expression of Transgenic Plants

Leaf painting assays: As plasmid pBI121 contains the P5CS gene, which encodes Δ¹-pyrroline-5-carboxylate synthetase (*P5CS*) and bar gene (herbicide resistant gene) as selectable marker, putative transgenic grape (*Vitis vinifera*) plants were treated with the recommended concentration of Basta herbicide (2.0 g L^{-1} Basta) to establish the expression of the bar gene. As used in other studies to identify transgenic plants in faba bean (Moemen *et al.*, 2005) and Chinese cabbage (Chaewan *et al.*, 2007). The bialaphos resistant plantlets were painted with Basta herbicide (bialaphos) to test their resistance as in other studies. Non transgenic plant leaves turned yellow then brown and dark (necrosis) within 2 days (Fig. 6), while the transgenic plant leaves remained green indicating their resistance to the herbicide (Figs. 6 and 7).

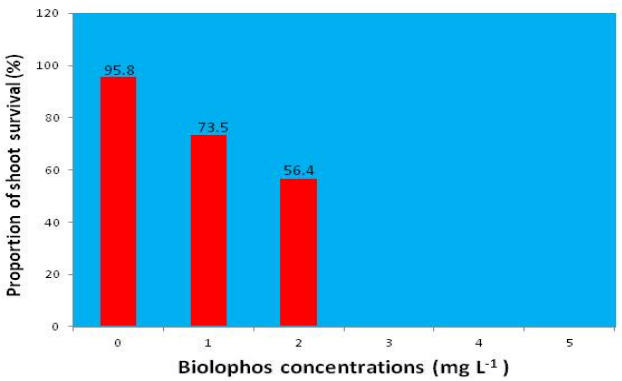

Fig. 5: Effect of bialaphos concentrations on leaf segments of grape cv. Thompson Seedless

Fig. 6: Leaf painting evaluation of transgenic grape plant (Right) compared to non-transformed grape plant (Left)

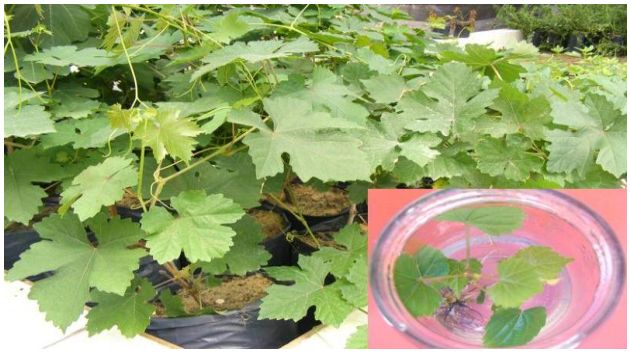

Fig. 7: Transgenic grape plants cultivar Thompson seedless containing *p5CS* gene

PCR detection of transformed grape plants: Genomic DNA was isolated from the transformed bialaphos resistant grape plants. PCR was used to confirm the integration of the *P5CS* gene into the genomic DNA of the putative transgenic bialaphos resistant tissues. In this study, the *P5CS* specific primers and bar specific primers were designed to amplify a fragment of 2100 bp and 540 bp, respectively (Fig. 8). Recombinant binary vector pBI121containings the *P5CS* gene was identified by double digestion with *BamH*I/*Sph*I (Fig. 8). Out of 14 leaf fragments 12 were recombinant as they showed a higher molecular weight fragment at 2100

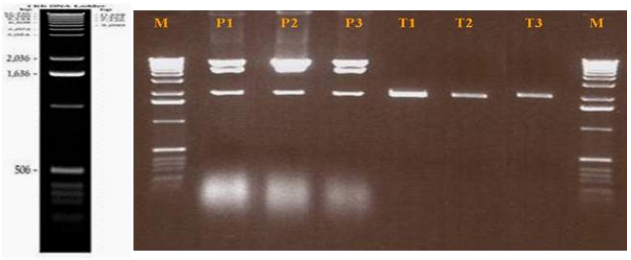

Fig. 8: Restriction digestion of the cloned fragment of *P5CS* gene in the binary vectorpBI121 under the control of the P35S promoter with *BamH*I and *Sph*I (Lanes P1-P3).PCR detection of *P5CS* gene into putative transgenic grape cv. Thompson seedless plant, amplifying 2100 bp (Lanes T1-T3). Lane M: DNA marker (1kb plus DNA ladder)

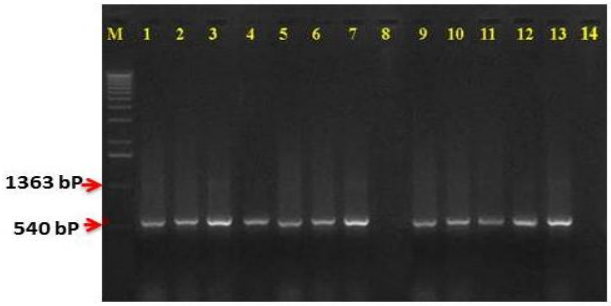

Fig. 9: PCR detection of *bar* gene in putative transgenic grape (*Vitis vinifera*) cv. Thompson seedless plants, amplifying about 540 bp with transgenic plants

bp in DNA from the plasmid carrying *P5CS* (lanes P1-P3) as a positive control and also from DNA purified from putative transgenic plants (Lanes T1-T3). A*P5CS* specific band (540 bp) was amplified from DNA extracted only from the transgenic grape plants but was absent in non-transformed plants (Fig. 9) 24% of the transformed lines that were positive for the *P5CS* gene were positive for the bar gene. These results confirmed that using *Agrobacterium*-mediated transformation was successfully applied to clone genes to grape.

Free proline level: Proline concentration was determined in transgenic and control plants in normal condition without the presence of any antibiotics (Table 3). The proline level produced by the constitutive expression of the *P5CS* transgene was over 6 times higher than the control and reached 1960 µg g^{-1} FW compared with 330 µg g^{-1} FW in control plants.

Discussion

To develop optimum conditions for genetic transformation the first perquisite is to develop efficient method for regeneration *in vitro* (Wędzony *et al.*, 2013; Verma *et al.*, 2014). In this study, leaf sections (0.5–1.0 cm) were cultured on NN basal medium combined with different concentration of growth hormones in order to optimize the best callus induction media. NN basal medium supplemented with 2.0 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} TDZ was the most effective for the induction of callus (92%) and shoot formation (+++). Several studies on *Vitis rupestris* (Olah *et al.*, 2003), *Vitis rotundifolia* (Dhekney *et al.*, 2011; Li *et al.*, 2014) and *Vitis vinifera* (Vidal *et al*., 2009) has been similarly succeeded to obtain regeneration using leaves as explants. In agreement with our results, Mullaw *et al.* (2010) reported that abundant embryogenic callus in wine grape was obtained from leaf and floral explants supplemented with 9.0 µM 2, 4-D +17.0 µM IASP in combination with either 1.0 µM BA or 1.0 µM TDZ in darkness**.** In contrast Nakajima *et al.* (2000) reported that the embryogenic callus from grape ovules was successfully produced on culture medium consisting of half strength MS supplemented with 1.0 µM 2,4-D and 0.2–5.0 µM TDZ. Also, the majority of the plantlets derived from micro shoot tips and nods of *Vitis pseudoreticulata* as obtained on half-strength MS solid medium supplemented with 9.0 µM TDZ and 2.9 µM NAA (Guan *et al*., 2013). Some studies on different plant species such Lingonberry leaves (Debnath, 2005) and Arabidopsis leaves (Guan *et al*., 2006) have reported good response with 2,4-D and TDZ to induce callus formation. The regeneration of plants from callus is often influenced by the choice of cytokinin for example, TDZ caused callus induction from hypocotyl or cotyledon explants of *Ochna integerrima* but applied BA did not induce callus (Ma *et al.*, 2011).

In the current study, shoot organogenesis frequency and shoot number per explant were significantly less when zeatin riboside or when TDZ was used in isolation whereas, shoot regeneration was detected when zeatin riboside was applied with TDZ. A series of experiments has demonstrated that regeneration success in grape can be induced efficiently by TDZ (and it has been found to be the most active cytokinin substitute for the hormonal requirement of somatic embryogenesis and morphogenesis for *in vitro* manipulation of many of woody species (Ola´h *et al*., 2003; Mullaw *et al*., 2010). Few studies have explained the mechanism of TDZ but it is suspected of promoting regulator morphogenesis in plantlets through the alteration of growth hormones, either cytokinin or auxin (Debnath, 2005) or by inhibiting cytokinin oxidase activity (Hare and Van-Stadan, 1994; Guo *et al.*, 2011). TDZ has been reported to be very efficient in stimulating adventitious shoot production in several recalcitrant woody plants (Janick *et al*., 1990; Gamage and Nakanishi, 2000; Pérez *et al*., 2000). Additional evidence for the influence of TDZ in combination with different auxins on the morphogenic potential of apples was recently provided by Xin *et al.* (2013). The results confirmed the stimulatory effect of TDZ in combination with different auxins or cytokinin on grape adventitious shoot regeneration.

Also, in our study, it was observed that 98% of the shoots produced adventitious roots in the rooting medium supplemented with 1.0 mg L^{-1} IBA and this medium can be considered as an optimized medium for root formation. In agreement with our results, Zhi-Ying *et al*. (2011) reported that 3/4 strength MS medium with the addition of 0.35 mg L^{-1} IBA was effective for root formation from regenerated adventitious buds in *Vitis vinifera*. IBA is commonly used in tissue culture procedures for various research on different plant species such as *Citrus sinensis* (Almeida *et al.*, 2002), *Centella asiatica* (Moghaddam *et al*., 2011), *Camellia sinensis* (Sirous *et al.*, 2012) and *Prunus empyrean* (Sadeghi *et al*., 2015) to stimulate cell enlargement, elongation and initiate roots from explants or callus. In multiple studies,rooted plant acclimatized readily to the greenhouse with survival rates of 85, 90 and 93% (Debnath, 2005; Fang *et al.*, 2007; Zhi-Ying *et al*., 2011) and these values agree with our data which showed 96% survival and normal growth and development.

A commonly used for selection of transformed shoots is bar and applied bialaphos (glufosinate ammonium) assay is used Our results showed that 3.0 mg L^{-1} was a lethal concentration of bialaphos and this is consistent with those obtained by Dedicova *et al.* (2015) who emphasis that the selection agent should completely suppress growth of untransformed plant cells. Herouet *et al.* (2005) indicated that the bialaphos resistance gene *bar* encode for the protein phosphinothricin acethyl transferase (PAT) that provides resistance to Phosphinothricin (PPT) by catalyzing the addition of an acetyl group to the free amino group and this applies to other herbicide derivatives such as bialaphos, which are competitive inhibitors of glutamine synthesis. In this study, 24 explants out of 100 survived on the bialaphos containing selection medium and were successfully transformed grapevine clones. This limited number of successfully transformed grapevine clones is not uncommon and has been attributed by various researchers to several different factor that influence the relative low transformation efficiency including initial difficulties experienced in the introduction of foreign genes and the selection and regeneration of transformed cells in grape species (Perl and Eshdat, 1998), escape genes during selection (Ogawa *et al.*, 2014), problems in the production of roots increasing the time to get plantlets (Luth and Moore, 1999), trouble in conditions required for successful Agrobacterium infection, (Li *et al.*, 2006) and also identifying the suitable *Agrobacterium* strain and selectable marker gene (Carimi *et al.*, 2012). Several methods for genetic transformation have been attempted for grape, but only Agrobacterium mediated transformation has been previously successful (Vivier and Pretorius, 2000).

In this study, recommended dose of Basta herbicide (2.0 g L^{-1}) was applied to weaned plantlets to prove the continued expression of the bar gene as a selectable marker. The data indicated that leaves of transgenic plant leaves remained green, while leaves of control plants turned and became necrotic yellow, which indicating resistance and sensitivity to the herbicide, respectively. In other studies of the T2 generation it has been found that transgenic plants can express different responses to the herbicide, for example only 48 out of 65 transgenic seedlings of maize remained Basta resistant (Chen *et al.*, 2008). Also for 130 explants in tobacco after selection with Basta herbicide subsequently only81 plants were Basta tolerant (62.30%) (Soliman *et al.*, 2009). Our results observed that only 24 out of 100 (24%) of putatively transformed grape plants with pBI121 contained the *P5CS* gene and were confirmed to be transgenic by PCR. In previous studies, researchers have attempted to focus on *P5CS* expression patterns in higher plants and they found increasing levels of constitutive transcript abundance compared with the genes *VaP5CS* (de Carvalho *et al.*, 2013), *OsP5CS* (Igarashi *et al.*, 1997), *PvP5CS* (Chen *et al.*, 2009), *JcP5CS* (Wang *et al.*, 2011), *AtP5CS1*(Zhuang *et al.*, 2011) *and MsP5CS1*(Fichman *et al.*, 2014). Induction of transcript expression of P5CS gene has also been reported in response to abiotic stresses (Neffar *et al*., 2011) and it was found to encourage proline synthesis and improved stress tolerance (Vendruscolo *et al.*, 2007; Patade *et al.*, 2013). This however depends on regulation of its transcript expression and further metabolism to meet the plant's needs (Sharma *et al*., 2011). This study indicated that, *P5CS* gene expression driven by this promoter led in 6.0 fold higher free proline when compared with non-transformed plants. The proline assay indicated that, the endogenous proline production is most likely the result of an increase in biosynthesis. There is a direct correlation between over production and accumulation of proline. This has been made in transgenic varieties that were engineered for overproduction of praline. In tobacco plants (Kavi-Kishor *et al*., 1995) and wheat (Sawahel and Hassan, 2002) expressing the *P5CS* gene led to an increase in proline content 14 and 12 times, respectively, compared to the non-transformed plants.

Because of proline biosynthesis is controlled by the activity of two P5CS (P5CS1 and P5CS2) genes in plants (Xue *et al.*, 2009) and the role of genes functioning in proline biosynthesis such as P5CS and catabolism such as proline dehydrogenases (PDH) in stress tolerance have been demonstrated in several plant systems (Mani *et al.*, 2002; Guerzoni *et al.*, 2014). We found that the transgenic plants which increased the expression of genes encoding the enzyme pyrroline-5-carboxylate synthase (P5CS) recorded high amounts of proline compared to non-transgenic plants. Our results were in agreement with Ashraf and Foolad (2007), who recognized proline as one of the most common amino acid accumulating as a result of disturbances in osmotic balance and suggest that it can serve as an indicator of salt stress and it is functional role is to defend cellular redox potential to stress conditions. Székely *et al.* (2008) indicted a positive correlation between accumulation of P5CS1 in chloroplasts under adverse conditions and increase in proline biosynthesis in plastids. The positive

correlation between proline accumulation and enhancement salt stress resistance provides a simple strategy for plants adapted to cope up with stress conditions and offers a possible biological marker beneficial in the identification and selection of salt resistant plants and plant cells Khorami *et al*. (2011).

Conclusion

An encoding gene (P5CS) was introduced into grapes plants by *Agrobacterium*-mediated transformation. Selection of different media and plant growth regulators are important for callus, shoot and root proliferation *in-vitro*. TDZ was found to be the most active cytokinin requirement for *in vitro* manipulation. Over expression of the abiotic stress *P5CS* gene enhanced synthesis of proline in transgenic plants and associated with providing abiotic stress tolerance.

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