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Development of high frequency *in vitro* shoot regeneration system from leaves of apple cultivar 'Oregon Spur' and optimization of antibiotics concentration

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Optimization of efficient shoot regeneration protocol and selection of transformed plants at optimum level of antibiotics are pre-requisite in genetic engineering. Here, we explored standardization of *in vitro* shoot regeneration protocol from leaf explants of apple cv. Oregon Spur for the first time using different concentrations of plant growth regulators. Further, we determined the effect of two antibiotics (kanamycin and cefotaxime) on shoot regeneration efficiency. About 95% of leaves induced adventitious shoots on MS medium supplemented with 4 mg/L BA and 0.2 mg/L NAA. The frequency of shoot regeneration increased to 100% when leaf explants were cultured on 2 mg/L TDZ along with 0.5 mg/L NAA. The number of shoots regenerated per leaf was enhanced but abnormal and vitrified shoots were obtained in case of TDZ supplemented medium. Dark incubation of initial one week decreased the shoot regeneration frequencies at all the combinations of plant growth regulators. Leaf explants were found highly sensitive to kanamycin and inhibited shoot induction above 5 mg/L. This concentration was found suitable for selection of transgenic shoots. Cefotaxime at 100-300 mg/L promoted organogenesis, whereas 500 mg/L was found optimal to eliminate Agrobacterial overgrowth after co-cultivation with leaf explants. Our results have demonstrated that the above protocol can successfully be applied for transgenic development in 'Oregon Spur' cultivar of apple in future trials.

Keywords: Cefotaxime, Kanamycin, *Malus domestica*

The 'Oregon Spur', a bud sport of delicious group of apples, is highly recommended spur cultivar in India. Fruit is usually blushed in dark red colour with medium to large size, and ripens prior to 'Starking Delicious'[1]. However, this cultivar is highly susceptible to diseases. Due to long breeding cycle, it is difficult to improve apple cultivars through conventional breeding. Hence, genetic transformation serves as an important and sustainable method for developing plant resistance to biotic and abiotic stresses. To generate transgenic plants, standardization of reproducible adventitious shoot regeneration protocol from the cultured cells or tissues is necessary. Regeneration method was first reported by Liu *et al.*[2] from different explant types excised from *in vitro* shoot cultures of apple 'Golden Delicious'.

Shoot regeneration is known to be influenced by the concentration and combination of auxins and cytokinins, explant orientation, medium composition, number of subcultures of the explant donors, dark/light incubation and genotype. Subsequently, efficient adventitious shoot regeneration protocols with shooting capacity of leaf explants have been established in different cultivars and rootstocks of apple[3-5]. Some of the above mentioned factors are considered in shoot regeneration experiments as they play an important role in increasing the efficiency of apple organogenesis. As the ability of *in vitro* plant regeneration in apple varies considerably with genotype, identification of other factors that remarkably affect the process of regeneration and transformation are extremely important[6]. To our knowledge, no previous research on *in vitro* shoot regeneration of apple cultivar Oregon Spur has been reported.

Agrobacterium tumefaciens mediated genetic transformation requires two steps for successful alteration and improvement of genome with desired traits. First step is co-cultivation of Agrobacterium and explants and elimination of bacterial overgrowth by the use of appropriate antibiotics. Second step is the regeneration and selection of transformed shoots on critical concentrations of antibiotics. During the transformation trials, a wide range of antibiotics like carbenicillin, vancomycin, augmentin, cefotaxime and timentin are commonly supplemented in culture media

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to overcome the excess bacterial growth seen on cocultivated tissues. Kanamycin and cefotaxime are the most frequently used antibiotics for genetic transformation process[7-10]. However, based on the genotype, some plant tissues show susceptibility towards the antibiotics, therefore, it is pre-requisite to evaluate the response of various antibiotics on plant tissue differentiation and growth before carrying out the transformation experiments. Antibiotics are not only toxic but may also inhibit shoot regeneration from the cultured tissues at certain levels[11,12]. Further, the low transformation efficiency could be due to the phytotoxic effect of the added antibiotics, because excessive dose inhibits the growth of transformed tissue and capacity of regeneration. The response of apple leaf explants towards the different levels of antibiotics is highly genotypic[11]. Therefore, the optimization of critical dose of antibiotics in post co-cultivation step is necessary before carrying out effective transformation experiments in 'Oregon Spur'.

Keeping the above aspects in view, here, we tried to establish an optimal combination of growth regulators and conditions for high frequency regeneration from *in vitro* leaves of the apple cultivar 'Oregon Spur'. In addition, we evaluated the influence of antibiotics on regeneration capacity and their concentrations was optimized to contain the Agrobacterium growth and for selection of regenerated shoots.

Materials and Methods

Source of plant material and media used

In vitro axillary shoots of apple 'Oregon Spur' were used as the source of explants for plant regeneration experiments. These shoots were raised through axillary bud culture and multiplied by enhanced axillary branching[13] in tissue culture laboratory of the Department of Biotechnology, Dr. YSP UHF, Nauni (H.P.). The shoots were maintained by sub-culturing at regular intervals on freshly prepared Murashige & Skoog[14] (MS) medium supplemented with 30 g/L sucrose, 8 g/L agar, 0.8 mg/L BA (6-benzylamino-purine), 1 mg/L GA_3 (gibberellic acid), 0.1 mg/L IBA (Indole-3-butyric acid) with pH of 5.7. About 30 mL of medium was poured into each of the 150 mL glass flasks. All the media were autoclaved at15 lbs/ln^2 at 121ºC for 20 min.

Adventitious shoot regeneration

Fully developed, apical and young leaves as explants were excised from one month old *in vitro* cultures of 'Oregon Spur'. Leaf petioles were removed, gently wounded across the veins and cultured on shoot regeneration medium (SRM) with their abaxial surface touching the medium. Shoot regeneration medium consisted of MS basal salts and vitamins, 30 g/L sucrose supplemented with benzyl adenine (1-4 mg/L) or thidiazuron (TDZ) (0.2-2 mg/L) along with naphthalene acetic acid (NAA) (0.2-1 mg/L) or IBA (1 mg/L) for a total of 26 combinations. After leaves were cultured on media, half of the culture flasks were incubated in 16 h photoperiod at a light intensity of 40 $\mu M/m^2/s$ at 25ºC±2. The other half was maintained in darkness for one week and then exposed to light. Each experiment consisted of 8 flasks each having 7-9 leaves, and was repeated three times. Data were recorded on percent regeneration of adventitious shoots and on number of regenerating shoots per responding leaf explant after 5 weeks of initial culture.

Multiplication, rooting and acclimatization of regenerants

Shoots (0.5-1 cm in length) regenerated on leaf explants in the above experiments were excised and propagated on MS multiplication medium for a total of three subcultures at an interval of 4 weeks. To induce rooting of the regenerated shoots, 2 cm long single shoots were placed on half strength MS medium containing 0.1-0.5 mg/L IBA. The multiplication rate and rooting frequency were observed after 4 weeks of culture. For acclimatization, *in vitro* rooted shoots were gently washed off all the adherents. These were then treated with fungicide (0.025% bavistin) for half an hour and transferred to pots containing cocopeat and vermicompost mixture in the ratio of 5:1. These plantlets were maintained in the greenhouse under high humidity.

Influence of antibiotics

Cefotaxime (cef) ranging from 100-500 mg/L and kanamycin (kan) from 1-25 mg/L were evaluated alone as well as in combination on the regeneration response of 'Oregon Spur' leaves using the same shoot regeneration process as mentioned above. In addition, optimal concentration of 'cef' and 'kan' were determined for elimination of bacterial growth and selection of putative transformants, respectively. For cocultivation, leaf explants were infected with *A. tumefaciens* strain LBA4404 bacterial suspension for 10 min and blotted on sterilized filter paper. These explants were then shifted to SRM supplemented with 100-500 mg/L cef alone to eliminate excess bacterial growth and combined with 1-5 mg/L kanamycin for selection of shoots. Leaves cultured on antibiotics free

medium were taken as control. Cultures were regularly checked for Agrobacterium overgrowth and its suppression. The explants which showed excess bacterial growth were washed in 100 mg/L cef solution, blotted again and cultured on the same selective medium. The percentage of contaminated explants was recorded during and after two weeks period.

Statistical analysis

The experiments were conducted using completely randomized design (CRD) for single factor experiment with each treatment in triplicates. The data were subjected to analysis of variance and differences among mean values were carried out according to Duncan's Multiple Range Test P <0.05 using SPSS software version 16.0 (SPSS Inc., Chicago, USA).

Results and Discussion

Reproducible and efficient shoot regeneration, rooting and acclimatization of apple 'Oregon Spur' were achieved in the present investigation using leaves as explants. In most of the apple cultivars and rootstocks, leaf explants have been used for regeneration of shoots and resulted in good regeneration capacities and number of regenerating shoots[5,15-17]. Type of plant growth regulators, and their dose added in the regeneration medium are important to enhance the regeneration capacity. A number of researchers have used high concentrations of BA and TDZ along with low concentrations of NAA and IBA for shoot regeneration in apple[4,18-20]. In the present study also, various combinations of these growth regulators were used for the standardization of shoot regeneration protocol.

Effect of BA with NAA on shoot regeneration

Fourteen different combinations of BA and NAA supplemented in MS medium were tested. At lower concentrations of BA (1.0-1.5 mg/L) with consistent level of NAA (0.5 mg/L), the leaf explants grew in size initially and calli appeared around the excised region and petiole, but regeneration was not observed even after eight weeks of incubation. Some adventitious buds and shoots started appearing on middle portion of leaves at higher concentrations of BA up to 4 mg/L. Very less callogenesis was observed at this concentration. Regeneration frequency was observed to increase with the increase in level of BA. Data acquired after five weeks of culture revealed the higher regeneration frequency of 90.84% and 3.46 average numbers of shoots per regenerating explant on medium supplemented with 4 mg/L BA and 0.5 mg/L NAA (Fig. 1A and Fig. 2 A & B). Similar concentrations of BA with low level of 0.2 mg/L NAA resulted in the highest level of regeneration ranging from 31% up to 95% with 4.76 healthy green shoots per leaf (Fig. 1B). Hence, enhanced regeneration frequency and mean number of shoots were obtained from explants cultured on low concentration of NAA (0.2 mg/L) with high level of BA (4 mg/L) making it the most efficient combination for inducing direct and highest organogenetic response (Fig. 2 A & B). The regenerated shoots continued to grow on the same medium up to 8th wk (Fig. 1C). The apple cultivars 'Red Chief', 'Royal Gala' and rootstocks MM106, M26, M7, B9 and 'fupingqiuzi' also resulted in almost similar regeneration frequencies from leaf explants on medium having BA and NAA during regeneration experiments[15,21]. In contrast, some researchers[3,18] obtained good shoot regeneration capacity from the leaf and internodal explants of apple cultivars at lower concentration of BA (0.5-2 mg/L) and NAA (0.2 -0.5 mg/L). However, in our study, the doses up to 1.5 mg/L BA inhibited the shoot induction while increasing concentrations promoted it.

Fig. 1 — (A & B) Direct shoot regeneration on different concentrations of BA with NAA; (C) Increased length of regenerated shoots on the same medium after 8th week; (D & E) Effect of dark treatment on shoot regeneration; (F) Regenerated shoots on the same medium after dark treatment; (G & H) Rooting of regenerated shoots on half strength rooting medium having different concentration of IBA; and (I) Hardening of regenerant in cocopeat and soil (1:1)

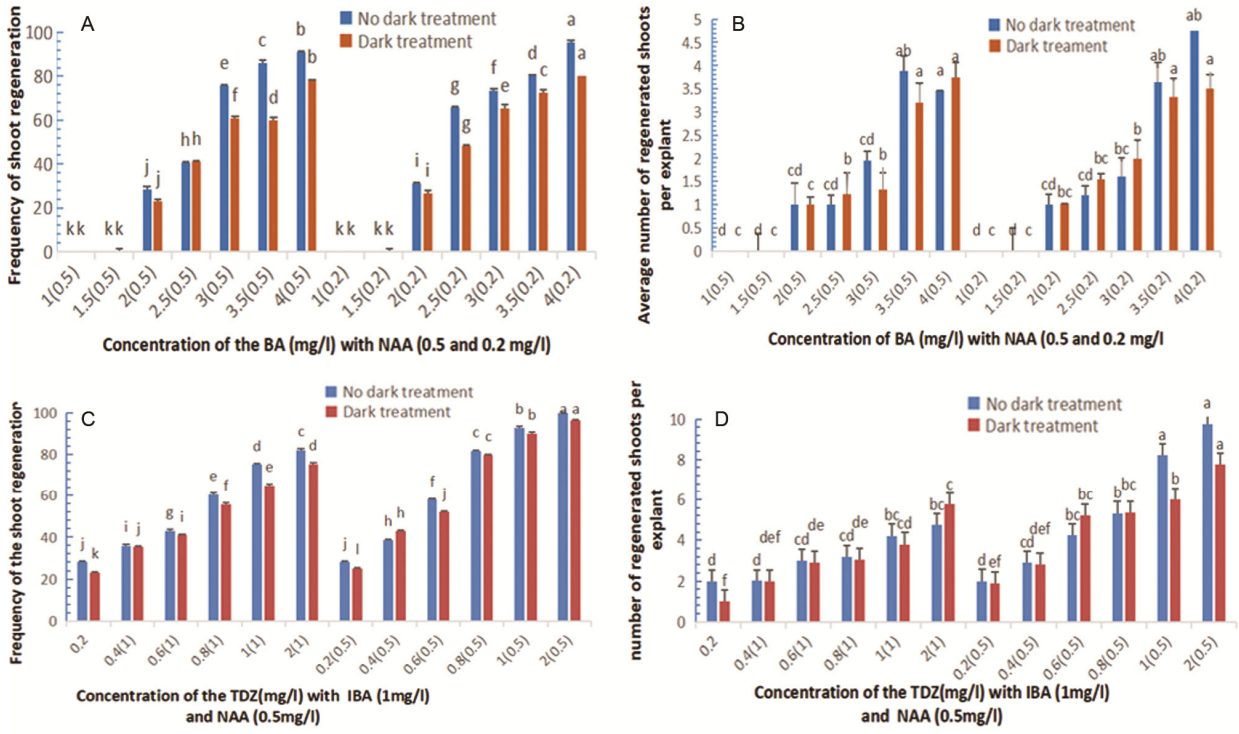

Fig. 2 — Effect of plant growth regulators (A & B) BA along with NAA (0.5 and 0.2 mg/L); and (C& D) TDZ along with NAA (0.5) and IBA (1 mg/L) with and without dark treatment on (A & C) shoot regeneration efficiency; and (B & D) average no. of shoots per explant [Data in the bar graphs are presented as means ± SE. Different letters indicate significant differences, as determined using Duncan's multiple range test ($P \leq 0.05$)]

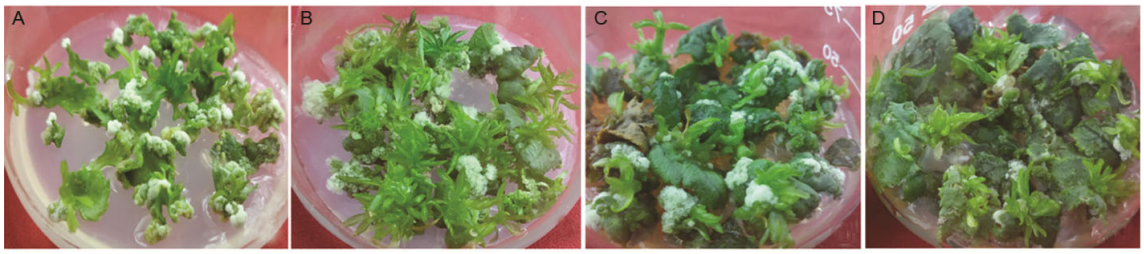

Fig. 3 — Effect of different concentrations of (A & C) TDZ and IBA (B & D) TDZ and NAA on shoot regeneration without (A & B) and with dark treatment (C & D)

Effect of TDZ with IBA/NAA

Leaf explants of 'Oregon Spur' showed different morphogenic response on twelve different combinations containing TDZ (0.2-2.0 mg/L) and IBA (1.0 mg/L) or NAA (0.5 mg/L). Here, callogenesis was observed first at the cut ends of most of the leaves from where the shoots arose indirectly. Origin of some direct shoots was also observed. The shoot forming frequency from explants cultured on medium with TDZ and IBA combinations varied from 28.05 to 82.09% and mean number of regenerating shoots per leaf ranged from 2.0 to 4.75. The highest value of 82.09% along with 4.75 countable average numbers of shoots per regenerating explant was obtained on medium containing 2.0 mg/L TDZ and 1.0 mg IBA (Fig. 3A and Fig. 2 C & D). Similar results were obtained by previous workers in apple 'Royal Gala', 'Freedom', 'Pingyitiancha' on similar level of TDZ and IBA[19,22].

When the IBA was replaced with the NAA, a significant increase in regeneration frequency and numbers of regenerating shoots per explant were observed. Here also, increasing concentrations of TDZ resulted in enhanced regeneration rate which varied from 28 to 100%. Maximum shoot induction of 100% and 9.75 average number of shoots per responding explant were observed on MS medium supplemented with 2.0 mg/L TDZ and 0.5 mg/L NAA (Fig. 3B and Fig. 2 C & D). Similarly, in some apple cultivars, higher

concentration of TDZ was required to induce 100% shoot regeneration from leaf explant[20,23,24]. However, in some cultivars like 'Golden Delicious', 'Gold Spur' and 'Bovery', low doses of TDZ was needed to yield higher percent regeneration[25].

TDZ and BA are the most frequently used cytokinins in apple shoot regeneration, and many studies showed comparison of both. In the present studies, TDZ was found equally effective at inducing high shoot regeneration and producing more number of shoots per leaf basis compared to BA. However, BA was found superior for inducing healthy and direct shoots, while all the TDZ combinations resulted in shoots with vitrification, compact rosette and stunted growth (Fig. 3). It was difficult to count the exact number of shoots formed on explant in some combinations. There was no increase in length of shoots till 8th wk of culture. Although, more potency of TDZ was observed in number of studies on apple for the regeneration of shoots from the leaves[22,26,27], but abnormal and vitrified shoot formation obtained was a major drawback in the present investigation. The variability in shoot regeneration frequency and morphology of shoots may be genotypic because different apple cultivars require certain concentrations and type of plant growth regulators to form healthy shoots. Different genotypes respond in different ways to cytokinins and auxins[11,20,28]. In many cases, TDZ was found more active than BA for shoot induction[5,27,29] but in a wide range of plants, it was reported to induce abnormal shoots[30,31] and cautioned because there are some documented cases of TDZ-induced abnormalities in apple *in vitro* culture such as hyperhydric and dwarf shoots, or shoot fasciation which supports the present results. The development of these abnormalities was reported to depend on the type of genotype and concentration of TDZ applied. da Silva & Dobranszki[32] suggested that the role of cytokinins needs improvement, because ineffective concentration might lie very near to effective levels, so a small deviation may cause a negative effect.

Effect of dark treatment on shoot regeneration

Incubation of leaf explants in the dark for one week decreased the regeneration frequency but trend remains the same in BA and NAA supplemented medium, as in case of light incubation. More callogenesis was recorded at the cut ends of leaf. About 78.22% leaf explants initiated 3.75 average number of shoots per responding explant on medium supplemented with 4 mg/L BA and 0.5 mg/L NAA (Fig. 1D and Fig. 2 A & B), a combination which resulted in 90.84% regeneration response without dark incubation. On reducing NAA concentration to 0.2 mg/L, frequency of regeneration varied from 26.55-80.02% and highest regeneration rate was observed on medium containing 4 mg/L BA and 0.2 mg/L NAA (Fig. 1E and Fig. 2 A & B). Number of shoots per leaf remained almost the same. The length of regenerated shoots did not increase much even after 8 weeks (Fig. 1F) as compared to shoots incubated in continuous light. Dark treatment during initial culture period is a key factor in plant regeneration. Our results are in line with the previous studies, that a number of apple cultivars resulted in direct shoot regeneration when leaf explants were incubated in the light condition[3,33]. Illuminated buds were able to accumulate reserve carbohydrates through photosynthesis that promoted bud formation over the long term[33], however, some cells may show differentiation in dark conditions[11]. Although in dark condition non degradation of NAA led to generation of more callus and decreased shoot induction in the present studies. On the other hand, increased regeneration frequency, number of shoots per explant and direct organogenesis were reported in number of studies on apple when leaf explants were incubated in dark initially for 1-3 weeks[5]. This difference in the regeneration response may be due to the genotypic effect and variation in duration of initial darkness to cultures.

Slight decrease in percent shoot regeneration, mean number of regenerating shoots and more callogenesis were observed after dark treatment in comparison to light, on medium supplemented with TDZ and NAA/IBA. Highest regeneration rate of 75.22%, and 5.75 average number of regenerating shoots per explant were observed on medium with 2 mg/L TDZ and 1.0 mg/L IBA (Fig. 3 C and Fig. 2 C & D). Whereas medium supplemented with combination of 2 mg/L TDZ and 0.5 mg/L NAA demonstrated shoot regeneration rate up to 96.20% (Fig. 3 D and Fig. 2 C & D). The average number of regenerating shoots per responding explant ranged from 1.89-7.75 in dark treated leaves. Previous workers reported that in some apple cultivars, light incubation delays the formation of calli from thin cell layers[2,19], and resulted in higher regeneration capacity in light as compared to dark[32]. Overall, higher regeneration rates were observed after dark incubation in TDZ supplemented media than that containing BA. However, regenerated shoots were closely packed, hyperhydric and poorly developed. Most of the shoots were originated from callus. Therefore, it is concluded that medium having 4 mg/L

BA and 0.2 mg/L NAA was chosen as the suitable medium for adventitious shoot regeneration from leaves demonstrating up to 95% regeneration rate when incubated in light.

Multiplication, rooting and hardening of regenerants

Once healthy and elongated shoots had been regenerated, they were easily multiplied on multiplication medium. About 95.45 and 83.78% rooting were recorded on medium having 0.1 and 0.2 mg/L IBA, respectively after one month of culturing (Fig. 1 G-H and Fig. 4 A & B). Rooting frequency decreased significantly with increase in concentration of IBA showing rooting rates up to 30.46% at 0.5 mg/L IBA. Callus was also induced at higher concentrations. However, Modgil *et al.*[34] and Kumar *et al.*[35] obtained 90-100% rooting efficiency in 'Malling 7' and 'Red Chief', respectively at 0.5 mg/L of IBA, while better rooting was reported in apple MM 111 rootstock when culture was incubated in darkness for initial few days on medium having 0.5 mg/L IBA following two step method[36]. Optimal IBA concentration necessary for rooting is genotype dependent and may possibly be due to the variation in endogenous auxin concentration[37]. The well rooted plantlets were successfully hardened (Fig. 1I). Survival percentage was recorded low when roots were developed through callus because callogenesis generally interferes with the acclimatization process.

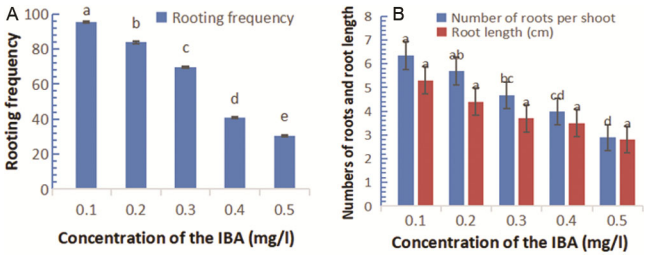

Fig 4 — Effect of different concentrations of the IBA on rooting ability of the regenerated shoots. (A) on rooting frequency; and (B) on number of roots and root length. [Data in the bar graphs are presented as means ± standard error. Different letters indicate significant differences, as determined using Duncan's multiple range test ($P \leq 0.05$)]

Effect of antibiotics on shoot regeneration response

Culture media having the antibiotic as selection agent make the selection of the transgenic shoots uncomplicated and regulated. Variations in the sensitivity to antibiotics were observed in various plant apple genotypes, cultivars and also among the different tissues and organs. Large number of escapes and chimeras were observed if the antibiotic concentration added in medium is kept low, but at higher dose, untransformed cells/shoots are killed and inhibition of the transformed cell growth are generally observed, which in turn delays the regeneration process of the transformed tissues. Antibiotic supplemented in the medium, its concentration and bacteriostatic elimination after transformation are the most crucial factors that affect the regeneration of shoots from transgenic calli[38]. Therefore, we have standardized these factors in the present studies prior to 'Oregon Spur' transformation experiments.

Effect of kanamycin

Among various doses of kanamycin added to the shoot regeneration medium. Shoot formation was observed only at 1-5 mg/L of 'kan' after six weeks of culture (Fig. 5 B-F, Fig. 6). The maximum shoot

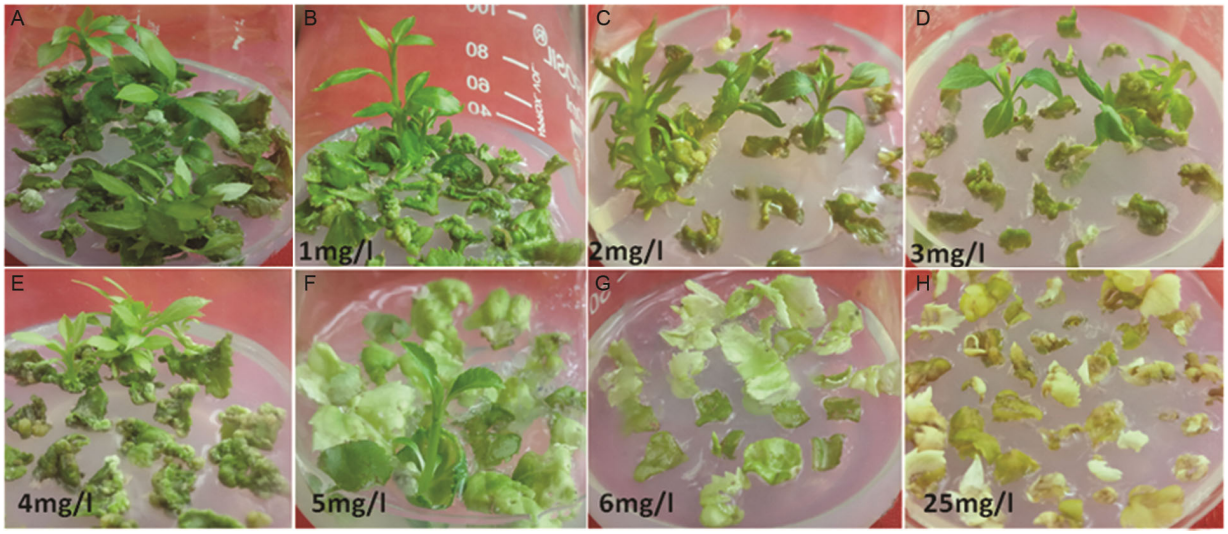

Fig. 5 — Effect of different concentrations of kanamycin on shoot regeneration from leaf explant (A) control, SRM without antibiotics (B-H) SRM supplemented with different concentration of kanamycin (1-25 mg/L kan)

regeneration frequency of 70.56% was obtained at 1.0 mg/L 'kan'. The regeneration rate decreased significantly up to 5 mg/L of 'kan' compared to control and resulted in 10% of the leaves inducing one shoot per regenerating leaf. It was further observed that leaves became yellow at various concentrations from 6 to 25 mg/L. There was no callogenesis and no shoot induction (Fig. 5 G-H). The kanamycin inhibitory effect led to decreased regeneration efficiency with the increase in concentration, and turning of 100% leaves to pale yellow may be due to the degradation of chlorophyll.

In most of the genetic transformation experiments, kanamycin was used as selection agent. However, it is considered as an inhibiting agent for inducing shoot regeneration from leaf explants of various apple genotypes and therefore, could be used in low concentrations[7]. Aminoglycoside-3-phosphotransferase encoded by *nptII* gene inactivates the kanamycin added to the medium by phosphorylation[39], which leads to selection of the transgenic shoots. Transgenic shoots selection efficiency with this antibiotic is highly dependent on the genotype and species[40], while dose is also a key factor in the present studies.

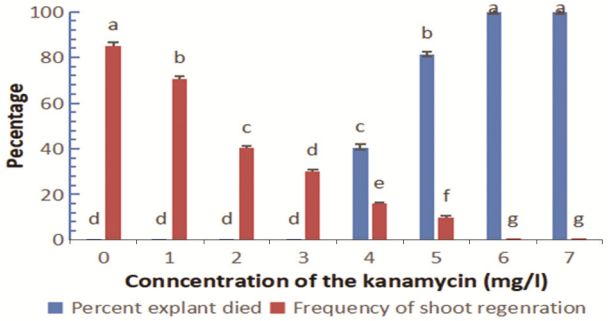

Fig. 6 — Effect of kanamycin on shoot regeneration efficiency from leaf explant. [Data in the bar graphs are presented as means ± SE. Different letters indicate significant differences, as determined using Duncan's multiple range test ($P \leq 0.05$)]

Effect of cefotaxime

Cefotaxime at the level of 100-500 mg/L was tested to evaluate the shoot regeneration efficiency. It has been observed that all the five concentrations revealed shoot induction. SRM containing 100 mg/L cef enhanced significantly the shoot differentiation from leaf explants to 80% with 3.1 mean number of regenerating shoots per explant. At higher concentration, leaf explants remained green and induced callus first. Regeneration frequency was reduced considerably to 75, 70.6, 30 and 15.8% with 1-2 no. of shoots per explant at 200 mg/L 300, 400 and 500 mg/L 'cef', respectively (Figs 7 and 8). Our results are in agreement with the previous workers that cefotaxime influenced plant morphogenesis either positively or negatively[9,41].

In the present study on 'Oregon Spur', plant regeneration was not much influenced by the lower concentrations of 'cef' up to 300 mg/L as compared to control. The results observed by James *et al.*[41] and Maheswaran *et al.*[17] in apple are matched with our study where low concentrations of 'cef' have a stimulatory effect on regeneration. But these concentrations cannot be used for adequate control of *A. tumefaciens* during transformation trials. β-lactam antibiotics strongly induce callus formation, but

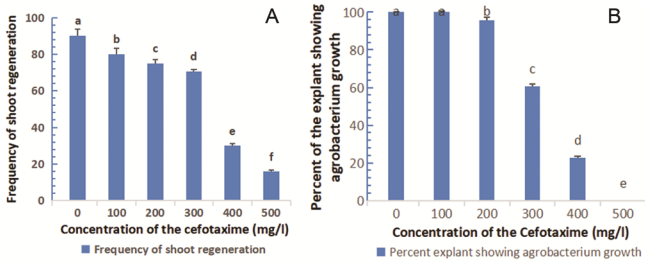

Fig. 8 — Effect of cefotaxime on shoot regeneration frequency and Agrobacterium growth. (A) shoot regeneration frequency; and (B) Agrobacterium growth after co-cultivation. [Data in the bar graphs are presented as means ± SE. Different letters indicate significant differences, as determined using Duncan's multiple range test ($P \leq 0.05$)]

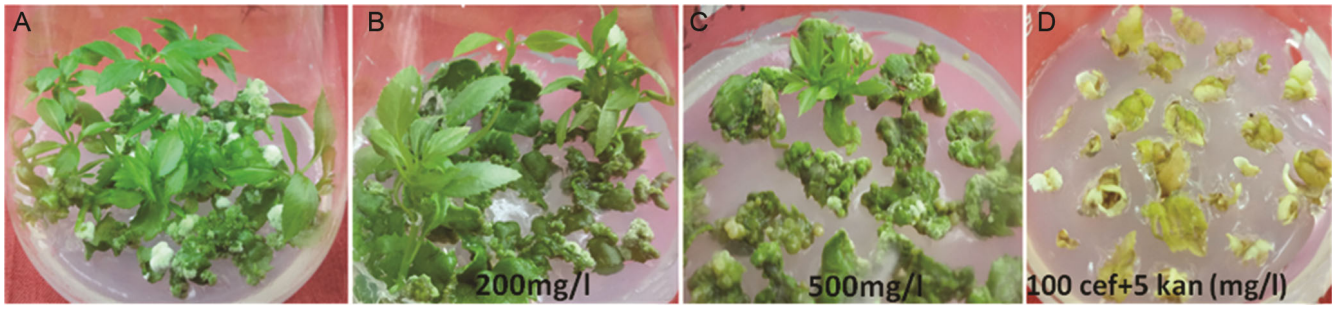

Fig. 7 — (A) Control SRM without the cefotaxime; Effect of cefotaxime (B) 200 and (C) 500 mg/L); and (D) cefotaxime (100 mg/L) + kanamycin (5 mg/L) on shoot regeneration from the leaf explant.

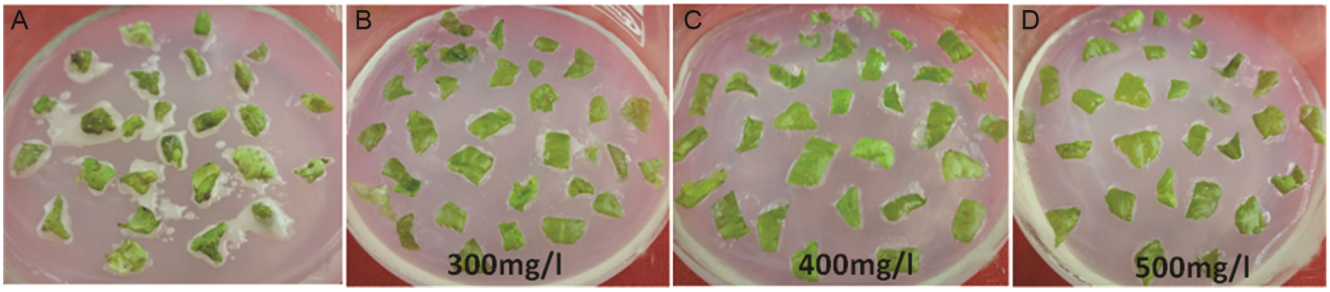

Fig. 9 — Bacteriostatic effect of cefotaxime on Agrobacterium growth after co-cultivation showing reduced over growth on higher concentration (A) control without antibiotics (B-D) SRM having different concentration of cefotaxime (300-500 mg/L)

inhibitory effect on shoot regeneration was observed at high concentrations[12]. Beneficial impact of 'cef' in enhancing regeneration and shoot development from apple leaves at lower concentrations (200-300 mg/L) has also been reported by previous authors[8,12] whereas, 500 mg/L led to abnormal growth with reduced regeneration frequency which supports the present results.

Combined effect of kanamycin and cefotaxime

While studying the combined effect of both the antibiotics on leaf morphogenesis of 'Oregon Spur', it has been observed that antibiotics drastically showed inhibitory effect on shoot regeneration. Medium having 1 mg/L 'kan' with 100-500 mg/L 'cef' resulted in callusing of leaves which became greenish yellow after 3 wk. However, significant reduction in callus induction was observed at 2 mg/L or more kan with 200-500 mg/L 'cef' (Fig. 7D). These antibiotics caused yellowing of leaves (chlorosis) earlier which later on turned pale white/brown at some portions, and became necrotic in all combinations of 'cef' and 'kan'. A direct correlation was observed between increased concentration of antibiotics and yellowing/ necrosis of leaf explants of 'Oregon Spur'. This may be due to the reason that both the antibiotics together exerted extra pressure on the cell growth and division which led to the necrosis of cells and ultimately death of explants. The present results are in agreement with Yepes & Aldwinckle[7] who also tested these combinations and did not observe regeneration in different apple cultivars and rootstocks.

Bacteriostatic effect of cefotaxime on Agrobacterium growth

The elimination of the excess agrobacterium growth from leaf explants is essential to save the explants and improve transformation efficiency during gene transfer. It may affect the regeneration of shoots if the Agrobactrium residuals remain within plant tissue and growth media[38]. There are a number of antibiotics used to eliminate agrobacterium growth during transformation trials, such as cefotaxime, augmentin, timentin, carbenicilin, hygromycin and geniticin. Among these, cefotaxime is a cephalosporin antibiotic which has a broad spectrum activity, inhibit bacterial cell wall synthesis, low eukaryote toxicity, and effectiveness at low doses. Therefore, it has frequently been used in agrobacterium mediated transformation experiments to avoid bacterial persistence in the medium after transformation. It has been used successfully to terminate overgrowth of agrobacterium in apple by many authors[8].

After evaluating regeneration ability of 'Oregon Spur' at 100-500 mg/L cef, it was also tested to control the agrobacterium growth. Uncontrolled bacterial growth of 95.7-100% was observed when infected leaves were co-cultivated at 100 -200 mg/L 'cef' supplemented medium. Whereas, the bacterial overgrowth was controlled to some extent at 300-400 mg/L cef (Fig. 8B and Fig. 9 B & C). It has been observed that 4-5 days after cocultivation, 60.6 and 22.7% of leaves showed bacterial growth at 300 and 400 mg/L 'cef', respectively, which needed blotting on every alternate day. Leaf explants did not show bacterial overgrowth needed to blot repeatedly at 500 mg/L 'cef' (Fig. 9D). Hence, this concentration was found to be the best because it showed strong bacteriostatic effect and completely eliminated the bacteria from infected leaf explants. Similar effect of 'cef' on morphogenesis has also been observed in other apple genotypes[8,41,42]. Hence, the present results suggest that 500 mg/L 'cef' can be used in medium immediately after cocultivation to inhibit the agrobacterium overgrowth. It can be reduced to 300 mg/L gradually after about 15 days to obtain the regeneration of putative shoots.

Conclusion

Above study reports identification of critical factors for enhancing shoot regeneration capacity of leaf

explants of apple cultivar 'Oregon Spur'. BA was found more effective cytokinin than thidiazuron (TDZ) for high quality shoot induction. Kanamycin at low concentration of 5 mg/L and cefotaxime at 500 mg/L can be used for selection of transformed shoots and to contain the agrobacterium persistence, respectively in the medium after transformation. The concentration of the cefotaxime should be lowered after 2-3 weeks to decrease the combined inhibitory pressure of the antibiotics on the regeneration efficiency. To the best of our knowledge this is first report of optimized protocol for genetic transformation of apple cultivar 'Oregon Spur', and can be used to generate transgenic plants of 'Oregon Spur' resistant to biotic and abiotic stress.

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Conflict of interest

Authors declare no competing interests.

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