Optimization of *in vitro* adventitious shoot regeneration from leaf explants of apple cultivar 'Red Chief'

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Abstract
Apple is the most important temperate fruit crop of India. The technique of adventitious shoot regeneration is considered a pre-requisite for apple transformation. Therefore, highly efficient in vitro shoot regeneration system was established in 'Red Chief', an important spur cultivar of apple. In vitro derived leaf explants were cultured on MS medium supplemented with different combinations of growth regulators to induce adventitious shoots. The frequency of shoot formation with BA and NAA combinations ranged from 26.47-96.35% with significant differences between the treatments. 5 mg/l BAP and 0.2 mg/l NAA were found to be the best treatment showing highest regeneration rate and 5.23 shoots per explant.

Leaf explants showed maximum regeneration frequency of 98-100% with more than 10 shoots per explant using 1.8 or 2 mg/l TDZ each with 1 mg/l IBA but produced vitrified and stunted shoots. Leaf explants collected from 20,35 and 40 days old cultures and dark incubation for 10 days prior to growth in light depressed the regeneration rate and number of adventitious shoots. The regenerated shoots were micropropagated through axillary branching on 0.8 mg/l BA, 0.5 mg/l GA_3 and 0.1 mg/l IBA. About 85% microshoots were rooted in auxin free medium with 10 days initial liquid culture containing 3 mg/l IBA. Rooted plantlets were acclimatized successfully.

Keywords: Apple, *in vitro* regeneration, 6-Benzylaminopurine, Thidiazuron.

Introduction

Apple belonging to the family Rosaceae, is grown in temperate and subtropical regions in various parts of the world. It is a fruit crop of significant economic importance which has gained importance among the consumers throughout the globe. India is the eighth-largest apple producer in the world with 2,265,000 tonnes from 305,000 ha harvested area (http://www.fao.org/faostat/en/). This fruit crop is threatened by premature leaf fall (*Marssonina* blotch) caused by *Marssonina coronaria*, which is now widespread worldwide including India.

It became a major production limiting disease during recent years with 40-75 percent incidence in the State of Himachal Pradesh. All the available commercial cultivars are susceptible to this disease. Among the various apple cultivars, 'Red Chief' is an important spur cultivar recommended in India. Its fruit size is medium to large, uniformly dark in colour, aromatic, juicy and sweet. It develops colour 15 days earlier than 'Starking Delicious'.

Traditional apple breeding is time consuming process due to the long juvenile period, long breeding cycle and large field space requirement as well as high level of heterozygosity. Therefore, transformation appears to be a better approach for genetic improvement of perennial fruit trees[6,14]. Our main aim is to setup RNAi strategy through novel innovation host induced gene silencing (HIGS) approach to control *Marssonina* blotch in 'Red Chief' apple. The success of gene transfer, however, requires the regeneration of whole plants from somatic tissues like leaf. Adventitious shoot regeneration is, therefore, necessary for this purpose. Development of an effective transgenic system largely depends on an efficient and reproducible method for shoot regeneration from cultured cells or tissues[3,13].

Adventitious shoot regeneration has already been described by different researchers for several apple cultivars[3,16,20,24,26,34] and reported different regeneration rates depending on the varieties or type of explant. In apple, regeneration of shoots was found to be affected by various important factors like species variability, explant type, age of cultures, leaf orientation, types and concentrations of plant growth regulators and the culture environment[3,8,24,29,35].

Thus, in this study, a simple and efficient system of direct shoot regeneration from *in vitro* leaf explants of apple cultivar Red Chief has been optimized for genetic transformation studies. For this ourpose, the effect of growth regulators, dark treatment, leaf orientation and explants excision time were studied.

Material and Methods

Plant material: Shoot proliferating cultures of apple cultivar Red Chief used as donor cultures in the present study were established[21] in tissue culture facility of Department of Biotechnology, Dr. YSP UHF (HP, INDIA)[21] and maintained by monthly subculture on standardized shoot multiplication medium consisting of MS (Murashige and Skoog)[31] salts and vitamins supplemented with 1mg/l BA, 0.5 mg/l GA_3 and 0.1 mg/l IBA, 30g/l sucrose and 0.8 percent agar. pH of medium was adjusted to 5.7 before autoclaving. The cultures were incubated in growth room at 25±2ºC under a 16-h light/ 8-h dark photoperiod.

Adventitious shoot induction from leaf explants: First 4 apical, green coloured, young and fully expanded leaf explants were excised from 4 week old cultures. Leaf edges and basal parts were cut away and leaf tissues were wounded on veins and across the midrib. Explants were then placed on regeneration MS medium with adaxial surface oriented upwards. To study the effect of plant growth regulators on adventitious shoot induction, regeneration medium containing different combinations of cytokinins e.g. 6-benzylaminopurine (1-5 mg/l) or thidiazuron (0.2-2.0) and auxins like napthalene acetic acid (0.5&0.2 mg/l) or indole-3-butyric acid (1mg/l) were evaluated. For initial pre-treatment in dark, the cultures were incubated for 10 days before exposure to light under the same photoperiod as used for multiplication.

Effect of leaf orientation on the regeneration efficiency was also studied by placing the leaves adaxial surface in contact with the regeneration medium. To test the best excision time of leaf explants, they were collected from 20, 30, 35, 40 days old cultures. The regeneration frequency of adventitious shoots and the average number of shoots per regenerating explant were recorded after six weeks of culture.

Regenerated shoot multiplication, rooting and acclimatization: Healthy and non-vitrified regenerated shoots were cut from the leaf explants and cultured on above mentioned multiplication medium for shoot elongation and multiplication. Two step rooting method [21] was used for rooting of the regenerated shoots. For this, 2-3 cm long shoots were induced to root by pricking at the base with the sterilized needle and dipped for 10 days in ½ MS liquid medium containing 3mg/l IBA and then transferred to auxin free ½ MS solidified medium. The percent rooting was calculated after four weeks. The well rooted shoots were transferred to the protrays containing cocopeat and vermicompost mixture in the ratio of 5:1 as standardised for axillary bud raised shoot rooting. Later, on cocopeat was replaced by soil: compost mixture (1:1).

Statistical Analysis Each treatment consisting of 30 leaves was repeated three times. The data were analysed by Analysis of Variance (ANNOVA) followed by CRD test (at $P_{0.05}$) using OPSTAT software. Mean values and standard errors were used in the tables. Percent data were subjected to arc sine transformation values.

Results and Discussion

In apple regeneration systems, different type of explants were reported to be used for adventitious shoot regeneration but the leaf explants were considered the best for *in vitro* shoot regeneration in most of the apple cultivars and rootstocks[26,27,29,40].

In the present studies, the effect of two types of cytokinin BA and TDZ combined with NAA or IBA respectively, dark incubation and age of explants were studied in 'Red Chief' and it was found that all these factors significantly influenced the regeneration frequency.

Effect of plant growth regulators on shoot regeneration: It has been seen that the different types of plant growth regulators and their concentration have different morphogenic response on the shoot regeneration from leaf explants of 'Red Chief'. At low concentration of BA (1-2 mg/l) with constant level of NAA (0.5 mg/l), the explants enlarged in size and showed slight callusing at the cut ends, but no regeneration of shoots was observed even after eight weeks.

With the increase in concentration of BA upto 5 mg/l, the leaf explants began to respond for shoot induction. The best regeneration frequency of 90.06% and 4.78 shoots per regenerating explant was achieved on medium supplemented with 5 mg/l BA and 0.5 mg/l NAA (Figure 1a). When the concentration of NAA was reduced to 0.2 mg/l, the regeneration frequency was further enhanced to 96.35% with more than 5 shoots per explant (Figure 1b, Table 1). Thus, high concentration of BA (5 mg/l) in combination with low NAA (0.2 mg/l) was the most effective combination in inducing maximum regeneration rate in 'Red Chief'.

It has further been observed that with the increase in BA levels from 2.5 to 5mg/l, there was an increase in regeneration rate with healthy, non-vitrified and direct shoot formation after six weeks of incubation. The elongation of the regenerated shoots was observed on the same medium till 8th week (Figure 1c). Similar *in vitro* shoot regeneration capacity from leaf explants of apple cultivar 'Royal Gala' and rootstocks MM106, M7 and B9 were observed on medium having combination of BA and NAA[24,28].

Jamil and Khan[17] achieved adventitious shoot formation from leaves and internodes in some of the apple cultivars on low concentration of BA (0.5-2mg/l) and NAA (0.2 -0.5 mg/l). However, in our study, the levels up to 2 mg/l BA could not induce shoots, but higher concentration above 2 mg/l of BA promoted shoot regeneration frequency.

In the present investigation, when leaf explants were cultured on medium supplemented with combinations of TDZ and IBA, different morphogenic response was observed.

Although shoot regeneration was obtained on all concentrations of TDZ (0.2-2.0 mg/l) each with 1 mg/l IBA, but the shoots produced were vitrified, stunted, rosette type and no elongation was observed even after the 8 weeks of culture. The callus formation was observed at the cut ends of leaves from where the shoots arose indirectly. The regeneration frequency increased with the increase in concentration of TDZ and maximum percent shoot regeneration (100%) was observed on the medium having 2.0 mg/l TDZ (Table 2).

The shoots were formed in bunches in some cultures where it was difficult to count the number of shoots per explant. The initiation of shoot induction was observed one week earlier in comparison to medium having BA and number of shoots per explant was also observed to be higher. While evaluating the effectiveness of TDZ in 'Red Chief', we observed 95-100 % shoot regeneration on medium having 1.8-2 mg/l TDZ each with 1mg/l IBA (Figure 1d and 1e). Similar regeneration rate was obtained in 'Pingyitiancha' apple cultivar on similar level of TDZ and IBA[18].

Mitic et al[26] obtained 95% regeneration frequency from leaves using very high level of TDZ (5 mg/l) along with low IBA (0.3 mg/l). On the contrary, low concentration of TDZ resulted in higher frequency shoot induction with high number of healthy shoots per regenerating leaf in apple 'Golden Delicious', 'Bovery' and 'Gold Spur'[9]. In various studies, TDZ was found more potent to induce adventitious shoots from the leaf explants[1,8,18,27]. On the contrary, production of abnormal and highly vitrified adventitious shoots in our study seems to be a drawback. The differences in shoot regeneration frequency and shoot morphology may be due to the different genotypes. It has been suggested that in many plant species, the biological activity of TDZ is comparable or sometimes even higher than that of the most active adenine type cytokinins.

Table 1
Effect of various combinations of NAA and BA on adventitious shoot regeneration from leaf explants of apple cv. Red Chief

S.N.	Plant growth regulators		Frequency of shoot regeneration	Average no. of shoots per explant
	BAP (mg/l)	NAA (mg/l)		
1	1	0.5	0.00 (0.00)	0.00(0.00)
2	1.5	0.5	0.00(0.00)	0.00(0.00)
3	2	0.5	0.00(0.00)	0.00(0.00)
4	2.5	0.5	26.47(31.08)	1.88(7.42)
5	3	0.5	55.49(48.25)	1.65(7.83)
6	3.5	0.5	70.94(57.20)	3.78(10.35)
7	4	0.5	79.84(62.89)	3.26(10.56)
8	4.5	0.5	86.47(68.59)	4.50(11.66)
9	5	0.5	90.06(72.54)	4.78(12.04)
10	1	0.2	0.00(0.00)	0.00(0.00)
11	1.5	0.2	0.00(0.00)	0.00(0.00)
12	2	0.2	0.00(0.00)	0.00(0.00)
13	2.5	0.2	30.87(33.66)	1.96(8.042)
14	3	0.2	68.57(55.84)	2.32(8.23)
15	3.5	0.2	76.15(61.45)	3.96(10.49)
16	4	0.2	79.68(62.79)	3.65(10.43)
17	4.5	0.2	88.74(69.97)	4.68(11.08)
18	5	0.2	96.35(78.93)	5.23(12.87)
$CD_{0.05}$			2.62	3.25
SE			0.91	1.13

Values in the parentheses are arc sine transformed values

Table 2
Effect of various combinations of TDZ and IBA on adventitious shoot regeneration from leaf explants of apple cv. Red Chief

S.N.	PGRs		Percent shoot regeneration	Average no. of shoots per explant	Type of shoot
	TDZ (mg/l)	IBA (mg/l)			
1	0.2	1	30.29(33.24)	3.67(10.02)	Vitrified
2	0.6	1	56.66(48.40)	5.35(13.14)	Vitrified
3	1.0	1	69.33(56.30)	5.89(12.47)	Vitrified
4	1.4	1	90.36(72.29)	7.85(16.09)	Vitrified
5	1.8	1	98.48(83.83)	>10	Vitrified
6	2.0	1	100(90.00)	>10	Vitrified
$CD_{0.05}$			4.95	3.45	
SE			1.59	1.396	

Values in the parentheses are arc sine transformed values

Similarly, in previous studies, high concentrations of cytokinin like TDZ and BA along with low concentration of NAA and IBA have been used for adventitious shoot regeneration studies in apple genotypes[1,26,27]. While comparing the two cytokinins in our investigation, BA containing medium resulted in direct organogenesis with longer shoots while TDZ developed shoots from intervening callus. In regeneration system of apple, both pathways for shoot induction have been reported, which depend upon genotype, cytokinin-auxin type and ratio. In cultivar Gala, indirect regeneration[9] was reported while in Jork 9 and MM106, shoots produced were direct[30,32].

In one of our recent reports, low level of TDZ (0.8mg/l) with 1mg/l NAA was more effective in stimulating high regeneration rate with 90 per cent of shoots originated directly in apple rootstocks MM111[27].

TDZ has been used to induce adventitious shoots in a number of Malus species and genotypes [22,33], however, it was reported to develop abnormal shoots in a wide range of plants[7]. Moreover, TDZ induced abnormalities like vitrified shoots, dwarf character or shoot fascination in apple. In some reports[19], it has been cautioned for its use, however, Magyar- Tabori et al[24] reported that development of these abnormalities depends on the genotype and TDZ concentration applied.

Da Silva and Dobránszki[5] suggested that the primary role of cytokinins in apple shoot regeneration needs to be improved because ineffective concentration might lie very close to effective levels, so a small deviation may cause an adverse effect.

Effect of explant age, dark incubation and orientation: Leaf explants excised from 30 days old donor multiple shoot cultures resulted in the highest shoot regeneration. Variations were observed in the number of adventitious shoots developed from donor shoots of different ages. Regeneration frequency in younger leaves which were excised from 20 days old donor culture was reduced to 78.02% with 2.62 shoots per regenerating explant. In our study, regeneration ability of the leaves depressed dramatically when age of donor shoots exceeds 30 days. The regeneration frequency decreased to 30% and 5.34% with only 1.65 shoot number per explant in case of leaves collected from 35 and 40 days old culture respectively (Table 3).

Here, most of the explants were covered with white nodular mass of cells. Previously, many authors found that the uppermost 2-4 young leaf explants of 20-30 day old culture were the best for obtaining good regeneration frequency and number of shoots per explant[11,23,29,38]. Williams and Maheswaran[39] reported that response of leaf explants for organogenesis is dependent on the development stage of tissue in many different plant species. Thus, 30 days old donor shoots proved to be the best source of explants in the present research on 'Red Chief'. Younger material is easy to form organs *in vitro*, which might be because they have more metabolically active cells with more suitable hormonal situation[10].

The incubation of the leaf explants in the dark for 10 days adversely affected the *in vitro* adventitious shoot regeneration. The dark treatment leads to callusing of the explants and lowers the regeneration rate to 60 percent and number of shoots to 2 on same medium which resulted in higher per cent shoot regeneration with light incubation. Similar results were obtained by Modgil et al.[29] Direct shoot regeneration in apple cultivar in the light condition was also observed by the Belaizi et al.[2]

On the contrary, many authors reported the increased regeneration frequency, more number of shoots per explant and direct organogenesis, when dark pre-treatment was given to the explants[4,18,26,36,37]. But in case of our study, the dark treatment leads to callusing of the explants, a decrease in the regeneration frequency and number of shoots per explant. The difference in the regeneration response from the dark pre-treatment to the explants may be due to the genotypic effect. Another factor we evaluated was the orientation of explants which is also considered to be one of the important factors in adventitious shoot regeneration in apple.

By placing the adaxial surface of leaf explants in contact with medium resulted in very low or no shoot regeneration and the leaf curled towards inside from the edges and very less surface area of the explants remained in contact with the nutrient medium. There is little callus formation at the cut ends of the leaves.

Table 3
Effect of age of the leaf explants on the *in vitro* shoot regeneration in apple cv. Red Chief

S.N.	Leaf age (No. of days)	Percent shoot regeneration	Average no. of shoots per explant	Shoot induction (after no. of days)
1	25	78.02(62.16)	2.62(9.29)	35
2	30	96.35(79.18)	5.23(13.13)	28
3	35	30.12(33.54)	1.65(7.82)	40
4	40	5.34(13.79)	1.00(3.86)	42
$CD_{0.05}$		4.38	3.86	
SE		1.32	1.16	

Values in the parentheses are arc sine transformed values

Figure 1: *In vitro* shoot regeneration in apple cultivar Red Chief a) Direct shoot regeneration on MS medium with 5 mg/l BA & 0.5 NAA (b) 5 mg/l BA & 0.2 mg/l NAA c) continued elongation of shoots on same medium till 8 wks d) 1.8 TDZ & 1 mg/l IBA e) 2mg/l TDZ & 1mg/l IBA f) multiplication of elongated regenerated shoots g) *In vitro* rooted regenerants after two step rooting method b) acclimatized plants

Similar findings were observed in other apple cultivars like 'Royal Gala'[16], 'Greensleeves'[25] and 'Golden Delicious'[26] where abaxial surface of the leaf explants oriented towards the medium resulted in higher shoot regeneration rates. On the contrary in apple cultivars 'Orine' [12] and 'Melrose' [26], the adaxial surface in contact with the medium resulted in the best shoot regeneration which may be due to different genotype and other factors like growth regulator combinations.

The regenerated shoots multiplied well on multiplication medium (Figure 1f) standardized in our earlier studies[21]**.** Around 85 percent adventitious shoots were rooted with a few secondary roots (Figure 1g). Similarly, a high percent rooting was observed in G.30 apple rootstock on medium having 3 mg/l IBA[15]. The well rooted plantlets of apple cultivar Red Chief were successfully acclimatized in pots in the green house conditions (Figure 1h) and showed 60 percent survival.

Conclusion

Thus, it may be concluded that a simple, reproducible and highly efficient shoot regeneration protocol in apple cultivar Red Chief has been optimized in the present research which can further be used effectively for regenerating shoots from leaf explants transformed by *Agrobacterium tumefaciens* containing RNAi constructs.

Acknowledgement

The financial assistance received from UGC-Rajiv Gandhi National fellowship and ICAR- Central assistance scheme of College of Horticulture, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P) - 173230 are highly acknowledged.

References

1. Bacha N., Darkazanli K. and Abdul-Kader A., Direct organogenesis and plantlet multiplication from leaf explants of *in vitro*-grown shoots of apple (*Malus domestica* Borkh.) cv.'Golden

delicious' and 'MM111' rootstock, *Fruit, Vegetable and Cereal Science and Biotechnology*, **3**, 28-34 **(2009)**

2. Belaizi M., Paul H., Sangwan R.S. and Sangwan-Norreel B.S., Direct organogenesis from internodal segments of *in vitro* grown shoots of apple cv. Golden Delicious, *Plant Cell Reports*, **9**, 471-477 **(1991)**

3. Bhatti S. and Jha G., Current trends and future prospects of biotechnological interventions through tissue culture in apple, *Plant Cell Reports*, **29**, 1215-1225 **(2010)**

4. Caboni E., Lauri P., Damiano C. and Angeli S., Somaclonal variation induced by adventitious shoot regeneration in pear and apple, *Acta Horticulturae*, **530**, 195–201 **(2000)**

5. Da Silva J.A. and Dobránszki J., Dissecting the concept of the thin cell layer: theoretical basis and practical application of the plant growth correction factor to apple, Cymbidium and chrysanthemum, *Journal of Plant Growth Regulation*, **33,** 881-895 **(2014)**

6. De Bondt A., Eggermont K., Druart P., De Vil M., Goderis I., Vanderleyden J. and Broekaert W.F., Agrobacterium-mediated transformation of apple (*Malus* x *domestica* Borkh.): an assessment of factors affecting gene transfer efficiency during early transformation steps, *Plant Cell Reports*, **13**, 587-593 **(1994)**

7. Dewir Y.H., Naidoo Y. and da Silva J.A., Thidiazuron-induced abnormalities in plant tissue cultures, *Plant Cell Reports*, **37**, 1451-1470 **(2018)**

8. Dobránszki J. and Teixeira Da Silva J.A., *In vitro* shoot regeneration from transverse thin cell layers of apple leaves in response to various factors, *Journal of Horticulture Science and Biotechnology*, **88**, 60–66 **(2013)**

9. Dufour M., Improving yield of adventitious shoots in apple, *Acta Horticulturae*, **280**, 51–60 **(1990)**

10. Famiani F., Ferradini N., Staffolani P. and Standardi A., Effect of leaf excision time and age, BA concentration and dark treatments on *in vitro* shoot regeneration of M. 26 apple rootstock, *Journal of Horticultural Science*, **69**, 679-685 **(1994)**

11. Fasolo F., Zimmerman R.H. and Fordham I., Adventitions shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars, *Plant Cell, Tissue and Organ Culture*, **16**, 75-87 **(1989)**

12. Gamage N. and Nakanishi T., *In vitro* shoot regeneration from leaf tissue of apple (cultivar 'Orine'): high shoot proliferation using carry over effect of TDZ, *Acta Horticulturae*, **520**, 291–300 **(2000)**

13. Gruchała A., Korbin M. and Żurawicz E., Conditions of transformation and regeneration of Induka' and Elista' strawberry plants, *Plant Cell, Tissue and Organ Culture*, **79**, 153-160 (**2004**)

14. Igarashi M., Ogasawara H., Hatsuyama Y., Saito A. and Suzuki M., Introduction of rolC into Marubakaidou [*Malus prunifolia* Borkh. var. Ringo Asami Mo 84-A] apple rootstock via *Agrobacterium tumefaciens*, *Plant Science*, **163**, 463-473 **(2002)**

15. Isutsa D.K., Pritts M.P. and Mudge K.W., A protocol for rooting and growing apple rootstock microshoots, *Fruit Varieties Journal*, **52**, 107–116 **(1998)**

16. James D.J., Passey A.J. and Rugini E., Factors affecting high frequency plant regeneration from apple leaf tissue cultured *in vitro*, *Plant Physiology*, **132**, 148-154 **(1988)**

17. Jamil A. and Khan I.A., Adventitious shoot development from leaves and stem segments of apple cultivars *in vitro*, *Sarhad Journal of Agriculture*, **16**, 39-43 **(2000)**

18. Jin W., Wang Y. and Wang H., Adventitious shoot regeneration from leaves of apple rootstock 'Pingyitiancha' (*Malus hupehensis* var. pinyiensis) and genetic fidelity of regenerated plantlets using SSR markers, *Canadian Journal of Plant Science*, **94**, 1345-1354 **(2014)**

19. Keresa S., Bosnjak A.M., Baric M., Jercic I.H., Sarcevic H. and Bisko A., Efficient axillary shoot proliferation and *in vitro* rooting of apple cv. Topaz, *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, **40**, 113-118 **(2012)**

20. Korban S.S., O'Connor P.A. and Elobeidy A., Effects of thidiazuron naphthaleneacetic acid, dark incubation and genotype on shoot organogenesis from Malus leaves, *Hort Science*, **67**, 341-349 **(1992)**

21. Kumari N., Kumar A. and Modgil M., *In vitro* establishment and proliferation of axillary buds of 'Red Chief' apple, a commercially recommended variety, *The Indian Journal of Agricultural Sciences*, Submitted **(2020)**

22. Li B.Q., Feng C.H., Hu L.Y., Wang M.R., Chen L. and Wang Q.C., Shoot regeneration and cryopreservation of shoot tips of apple (*Malus*) by encapsulation–dehydration, *In Vitro Cellular and Developmental Biology-Plant*, **50**, 357-368 **(2014)**

23. Lu C.Y., Nugent G. and Wardley T., Efficient, direct plant regeneration from stem segments of chrysanthemum (*Chrysanthemum morifolium* Ramat. cv. Royal Purple), *Plant Cell Reports*, **8**, 733-736 **(1990)**

24. Magyar-Tábori K., Dobránszki J., da Silva J.A., Bulley S.M. and Hudák I., The role of cytokinins in shoot organogenesis in apple, *Plant Cell, Tissue and Organ Culture*, **101**, 251-267 **(2010)**

25. Maheswaran G., Pridmore L., Franz P. and Anderson M.A., A proteinase inhibitor from *Nicotiana alata* inhibits the normal development of light-brown apple moth, *Epiphyas postvittana* in transgenic apple plants, *Plant Cell Reports*, **26**, 773-782 **(2007)**

26. Mitic N., Stanisic M., Milojevic J., Tubic L., Cosic T., Nikolic R. and Ninkovic S., Optimization of *in vitro* regeneration from leaf explants of apple cultivars Golden Delicious and Melrose, *Hort Science*, **47**, 1117-1122 **(2012)**

27. Modgil M. and Pathania S., Plant regeneration from leaf explants in apple rootstock MM111: Effect of cytokinins and auxins, *Indian Journal of Biotechnology*, **17**, 370-378 **(2018)**

28. Modgil M., Guleria N., Ghani M. and Sharma J.N., Identifying somaclonal variants of the apple rootstock Malling 7 resistant to white root rot, *Scientia Horticulturae*, **137**, 148-155 **(2012)**

29. Modgil M., Handa R., Sharma D.R. and Thakur M., High efficiency shoot regeneration from leaf explants of *in vitro* grown shoots of apple, *Acta Horticulturae*, **696**, 123-128 **(2005)**

30. Modgil M., Sharma D.R. and Bhardwaj S.V., Micropropagtion of apple cv. Tydeman's Early Worcester, *Scientia Horticulturae,* **81**, 179-188 **(1999)**

31. Murashige T. and Skoog F., A revised medium for rapid growth and bioassay with tobacco tissue culture, *Physiologia Plantarum,* **15**, 473-479 **(1962)**

32. Pawlicki N. and Welander M., Adventitious shoot regeneration from leaf segments of *in vitro* cultured shoots of the apple rootstock Jork 9, *Journal of Horticultural Science,* **69**, 687-696 **(1994)**

33. Podwyszyńska M. and Cieślińska M., Rooting shoots of apple varieties and their tetraploids obtained by the *in vitro* technique, *Acta Scientiarum Polonorum: Hortorum Cultus*, **17**, 49–62 **(2018)**

34. Predieri S. and Malavasi F.F.F., High frequency shoot regeneration from leaves of the apple rootstock M26 (*Malus pumila* Mill.), *Plant Cell, Tissue and Organ Culture,* **17**, 133-142 **(1989)**

35. Rao C., Goh C. and Kumar P.P., High frequency adventitious shoot regeneration from excised leaves of Paulownia spp. cultured *in vitro*, *Plant Cell Reports,* **16**, 204-209 **(1996)**

36. Seong E.S., Song K.J., Jegal S., Yu C.Y. and Chung I.M., Silver nitrate and aminoethoxyvinylglycine affect Agrobacterium-mediated apple transformation, *Plant Growth Regulators,* **45**, 75–82 **(2005)**

37. Sun C.Y., Wang Y., Xu X.F., Sun Y., Zhu L.H. and Han Z.H., Regeneration from leaf segments of *in vitro*-grown shoots of *Malus baccata*, *New Zealand Journal of Crop and Horticultural Science,* **36**, 233-238 **(2008)**

38. Welander M., Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees, *Journal of Plant Physiology*, **132**, 738-744 **(1988)**

39. Williams E.G. and Maheswaran G., Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group, *Annals of Botany*, **57**, 443-462 **(1986)**

40. Zhang X., Qin Y., Liang D., Zou Y. and Ma F., Enhancement of *in vitro* shoot regeneration from leaf explants of apple rootstock G. 41, *In vitro Cellular and Developmental Biology-Plant*, **50**, 263-270 **(2014)**.

(Received 30th April 2020, accepted 08th July 2020)