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In vitro shoot regeneration and shoot tip necrosis in prunus necrotic ring spot virus free peach cv. July Elberta

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Abstract
The studies were conducted to develop a protocol for regenerating prunus necrotic ring spot virus (PNRSV) free shoots in peach cv. July Elberta using axillary buds as explants but *in vitro* propagation technique encountered serious problems due to high incidence of shoot tip necrosis (STN) during regeneration of peach cv. July Elberta. Axillary buds from field grown plants were used as explants and cultured on MS supplemented with different concentrations of BA and GA_3. The findings revealed that the synergistic effect of combination of BA (0.5 mg/l), GA_3 0.1 mg/l with casein hydrolysate (100 mg/l) or malt extract (100 mg/l) proved to be better for shoot regeneration. Frequent subculturing of nodal explants from *in vitro* derived shoots increases the number of multiple shoots, but the regenerated shoots exhibited the symptoms of shoot tip necrosis (STN). The findings of the present studies have clearly indicated that various factors such as strength of the media, different carbon sources activated charcoal and different calcium sources need to be investigated for reducing the incidence of shoot tip necrosis during *in vitro* shoot regeneration in tissue culture of peach cv. July Elberta.

Keywords: *In vitro* shoot regeneration, peach, shoot tip necrosis, virus

Introduction
Tissue culture techniques are becoming increasingly popular as alternative means of plant vegetative propagation. In recent years, micro-propagation is increasingly used for rapid clonal multiplication of several economic plants, restoration of vigour and yield lost due to infection and preservation of germplasms. In a relatively short span of time and space, a large number of plants can be produced starting from a single individual. *In vitro* propagation has shown promises for rapid and large scale clonal multiplication of disease-free planting material throughout the year. However, differences exist among all genotypes for their requirement of basal medium and growth regulators. The ability to establish auxillary bud cultures, proliferate shoots, induce rooting and to acclimatize the resulting plantlets are stages of *in vitro* propagation (Bell and Reed 2002) [1].

The use of tissue culture for temperate fruit and nut tree species have increased substantially since the early 1970s and virtually all temperate fruit tree species have been micropropagated with various degrees of success. The necessity to modernize the planting material production technologies of peach has been stimulated by many considerations such as the trends towards increasing the planting densities in field grown trees and the transition to intensive growing systems that includes the selection of new parents for breeding programmes, development and introduction of new cultivars and the modernization of tree habit and pruning. All these changes have created a demand for more and more quantities of quality planting material. The conventional system of propagation is not only time consuming but the material raised is neither uniform nor healthy. Application of *in vitro* techniques in temperate fruit growing can, therefore, be a viable alternative to circumvent these problems. Besides to the benefits of traditional grafting, *in vitro* raised shoot tips can be an efficient means of regenerating plant material free of endogenous microbial contaminants particularly graft transmissible pathogens (GTPs) like viruses, viroids and phytoplasma with enhanced potential for true-to-type cloning mature plants (Zilkah *et al*. 2002) [2].

Raising plantlets under *in vitro* conditions has several advantages both for production and research. *In vitro* micropropagation has often been applied for the improvement and rejuvenation of several tree species (Canon *et al*. 2006; Fabiana *et al*. 2006) [3, 4], virus elimination (Bisognin *et al*. 2008; Ribeiro *et al*.) [5, 6], study of physiological connections

between rootstocks and scions such as incompatibility, root to shoot communication or transport and use in quarantine as this method has a minimum risk for importing plants (Nelson 2004 ; Bortolotti *et al*. 2005) [7, 8]. Due to the multiple uses and advantages of micropropagation, this technology is of potential practical value and will contribute to the practical utility of micropropagation as a tool in temperate fruit tree biotechnology.

Materials and Methods

Survey and collection of plant material:

Surveys were conducted in the Experimental Earm of the Department of Fruit Science of Dr. Y S Parmar university of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh to ascertain the sanitary status of these plants in order to identify the peach cv. July Elberta plants free from PNRSV. Apparently healthy plants expressing no symptoms of PNRSV were used for drawing explants.

Serological virus indexing of mother plants

Serological indexing of true to type, apparently healthy trees of peach cv. July Elberta marked during surveys in the selected orchard was carried out for the selection of PNRSV plants by using standardized DAS-ELISA protocol Clark and Adams (1977) [9]. DAS-ELISA was performed by using the ELISA kit procured from BIOREBA, Switzerland. Leaf samples were processed and subjected to DAS-ELISA protocol.

Preparation of nutrient medium

The medium used for culturing axillary and terminal buds consisted of major and minor elements of MS (Murashige and Skoog 1962) [10] with the addition of different supplements. Plant growth regulators were prepared fresh each time. MS salts, vitamins and different combinations of growth regulators were mixed proportionately to get the desired concentration. Besides these, meso-inositol (100mg/l) and sucrose (3 percent) were added to medium and gelled with 0.8 percent Difco bacto agar (solidifying agent). Specific concentration of different growth regulators were added on to the media. Before adding agar, pH of the medium was adjusted to 5.6-5.8 with the help of 1N sodium hydroxide (NaOH) and 1N hydrochloric acid (HCL). After mixing agar by boiling, 10 ml medium was dispensed in 2.5×10.0 cm culture tubes and 30-35 ml in 64 × 105 mm (100 ml volume) Erlenmeyer flasks and plugged with non-absorbent cotton wrapped in muslin cloth (cotton plugs). The medium was then sterilized in autoclave at pressure 15lbs/inch^2 and temperature 121°C for 15-20 minutes.

Excision of axillary/terminal buds

Terminal/axillary buds ranging in size 0.2-0.5 cm were excised with the sharp scalpel blade and collected in distilled water. Terminal portion of the forced shoots of peach cv. July Elberta having 2-3 nodes of about 1-2 cm long were excised after removing their leaves. Before disinfection, beaker containing buds was covered with muslin cloth and kept under running tap water for about 1 hour. All buds were washed with Teepol using 2-3 drops in 100ml water and agitated strongly to wash out the debris, if any.

Surface sterilization

Washed buds were taken to laminar flow chamber and surface sterilized in order to find out the best surface sterilant. The explants were first treated with bavistin (0.2 percent) dissolved in sterile distilled water for 15-20 minutes. After draining it off, explants were washed 2-3 times with sterile distilled water. Then the explants were dropped into 70 percent ethanol for 40-60 seconds. Smaller ones were treated for 40-45 seconds and larger for 60 seconds. The buds were then surface sterilized with two different sterilants, sodium hypochlorite (NaOCl) @ 0.2 percent and mercuric chloride ($HgCl_2$) @ 0.1 percent in two treatments i.e. explants were treated with 2 percent sodium hypochlorite for 15-20 minutes and 4 percent for 10-15 minutes and then rinsed with sterile distilled water several times and kept until excision of meristems. After treating with sterilant and washing with sterile distilled water, the explants were placed on sterile petriplate lined with moist filter paper.

Establishment of buds

Axillary buds were cultured on MS (Murashige and Skoog 1962) [10] medium supplemented with salts and vitamins and 30g/l sucrose in addition to different concentrations and combinations of growth regulators i.e. BA (0.1-0.5 mg/l), GA_3 (0.1-0.5 mg/l) and ascorbic acid (50mg/l) for initiation. The media was solidified with 8g/l agar. All cultures were maintained and incubated at 25±2^{0}C for 16 hour light illuminated by 40 watt fluorescent tubes and 8 hours of darkness till their establishment. Contaminated cultures were removed from incubation room. After 3 weeks, viable cultures showing growth of leaves were transferred onto newly prepared MS medium of same composition or of different composition for further growth of stems and axillary shoots. Each surviving culture was labelled so that it could be traced back to the original shoot tip. The data on explant contamination and explant establishment on medium with different growth regulator combinations were recorded after 3 weeks of culturing. The effect of time of the year, position of buds on shoots and size of the explants on their establishment and proliferation were also taken into consideration. Shoots were maintained and their size and number were increased by subculturing the proliferated buds on MS medium supplemented with different growth regulators i.e. BA (0.1-1.0 mg/l), GA3 (0.1-0.9 mg/l) combined with 100mg/l each of Casein hydrolysate, yeast extract, fructose and Malt extract. Rate of shoot multiplication and length was recorded after 4-5 weeks in each treatment. The rate was determined by counting the number of new shootlets produced per shoot.

Cultural conditions

All *in vitro* cultures were maintained in a temperature controlled room at 25 ± 2 ^{0}C. Light consisted of white fluorescent tubes and an incandescent lamp and the intensity of light at the level of cultures was 4000 lux with a Photoperiod of 16 hours of light and 8 hours of darkness.

Virus indexing of *in vitro* established shoots

Virus indexing of *in vitro* established shoots was carried out using the same procedure as described earlier. For indexing of established shoots, leaf samples were taken from multiplied shoot clusters, raised from the buds. Rest of the shoots in these clusters were maintained on the same medium.

Shoot multiplication of virus tested shoots

After indexing, the shoots which were found free from PNRSV were taken further for shoot multiplication on the MS medium with best combinations standardized as above. The shoots testing positive in DAS-ELISA results were discarded.

Results

In vitro production of virus free planting material

In vitro technique involving axillary and terminal buds was used to produce PNRSV free peach cv. July Elberta plants. The detailed results for *in vitro* production of virus free plants is presented in the following paragraphs.

Serological virus indexing of mother plants:

DAS-ELISA based serological indexing of peach cv. July Elberta trees marked on the basis of visual symptoms in the Experimental Farm of Department of Fruit Science of Dr Y S Parmar University of Horticulture and Forestry was conducted and trees testing negative for PNRSV were further used as mother plants to draw explants for *in vitro* culturing to obtain virus free plantlets.

Initiation and establishment of peach cv. July Elberta buds:

Axillary and terminal buds of (0.2-0.3 cm) were excised from dormant shoots of the PNRSV indexed marked peach cv. July Elberta trees and brought to the tissue culture laboratory of the Department of Biotechnology, Dr YS Parmar University of Horticulture and Forestry Nauni. Excised buds were sterilized as per the standard procedure and later shifted to culture room and surface sterilized under the laminar airflow hood using 70 percent ethanol for 30 seconds and two sterilizing agents NaOCl (0.2 percent) and $HgCl_2$ (0.1 percent). It is evident from the data in (Table1) that $HgCl_2$ (0.1 percent) was a better sterilant than NaOCl as it resulted in less number of contaminated cultures (6 out of 25 buds) inoculated and had a highest percent survival rate of 76 percent as compared to 44 percent in case of NaOCl.

Table 1: Effect of sterilants on survival of inoculated buds

Sterilant (%)	Time (minutes)	No. of buds inoculated	No of contaminated cultures	Percent survival of buds
NaOCl (0.2)	10	25	14	44
$HgCl_2$ (0.1)	3	25	06	76

For *in vitro* establishment of peach cv. July Elberta, MS medium supplemented with different concentrations and combinations of two growth regulators (BA and GA_3) were used with 50mg/l ascorbic acid added to each combination. It is evident from Table 2 that BA and GA_3 successfully helped in establishing buds of peach cv. July Elberta under *in vitro* conditions. Out of the 4 combinations used for the establishment of peach cv. July Elberta buds, combination 4 comprising of BA 1.0 mg/l with 0.9 mg/l of GA_3 resulted in better establishment (60 percent) in comparison to all other combinations as 15 buds were established successfully from 25 inoculated cultures and the minimum bud establishment of 40 percent was recorded in combination 1 (0.3 mg/l BA and 0.2 mg/l GA_3). This combination resulted in the establishment of only 10 out of 25 buds inoculated (Figure1).

Table 2: Effect of different combinations and concentrations of growth regulators on MS medium on bud establishment

Combinations	MS medium supplemented with(mg/l)		No. of buds established/ No. of buds inoculated	Percent bud establishment
	BA (Benzyl adenine)	GA_3 (Gibberellic acid)		
1	0.3	0.2	10/25	40
2	0.5	0.5	12/25	48
3	0.8	0.7	12/25	48
4	1.0	0.9	15/25	60

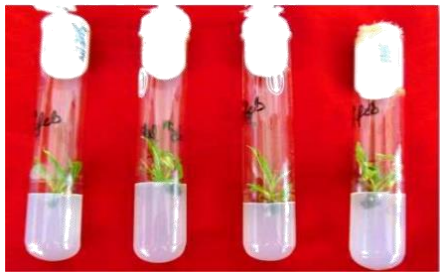

Fig 1: Healthy cultures of peach cv. July Elberta

Virus indexing of *in vitro* established shoots

Peach cv. July Elberta shoots were used for drawing leaves to conduct serological indexing using DAS-ELISA to record the status of PNRSV. Healthy leaflets from 10 established plants (3 weeks old) were removed from culture tubes under laminar airflow to avoid contamination and indexed by DAS-ELISA (Figure 2). Serological virus indexing is an essential step for *in vitro* production of virus free plants as it helps in eliminating infected cultures at an early stage. Indexing of *in vitro* established shoots in these studies was also conducted to know the status of PNRSV. OD values recorded in DAS-ELISA test clearly indicate that *in vitro* raised shoots were free from PNRSV infection as all established cultures had OD values less than twice the value of negative control (Table 3).

Table 3: Das-Elisa indexing of *in vitro* established cultures

Plant No	OD Values (A_{405}nm)	Status of PNRSV
1	0.098	Negative
2	0.102	Negative
3	0.117	Negative
4	0.054	Negative
5	0.087	Negative
6	0.110	Negative
7	0.105	Negative
8	0.066	Negative
9	0.050	Negative
10	0.109	Negative

Fig 2: DAS-ELISA test of *In vitro* established peach cv. July Elberta

***In vitro* shoot multiplication of virus tested established shoots**

After indexing, virus free *in vitro* established buds were further sub-cultured for shoot multiplication on MS basal medium supplemented with BA and GA_3 growth regulators. Plantlets were transferred to four different combinations having different concentrations and combinations of growth regulators along with additives. After 2 weeks, shoot multiplication initiated in a few cultures. Data set out in Table 4 revealed that combination 1 (BA 0.5 mg/l, GA_3 0.1 mg/l with casein hydrolysate 100 mg/l) and combination 4 (BA 0.5 mg/l, GA_3 0.1 mg/l with malt extract 100 mg/l) proved to be better for shoot regeneration than the remaining two combinations as both these combinations resulted in shoot regeneration up to 2 cm. However, shoots in all combinations turned brown due to necrosis and the cultures failed to survive despite the addition of additives like Casein Hydrolysate and Malt extract (Figure 3).

Table 4: Effect of different concentration and combination of growth regulators on the *in vitro* multiplication of shoots

	MS medium supplemented with (mg/l)			Multiplication	Length of
Combination	**BA**	**GA_3**	**Additional supplements (100 mg/l)**	**rate**	**shoots (cm)**
1	0.5	0.1	Casein Hydrolysate	1:2	2.0
2	0.5	0.1	Yeast extract	1:1	1.5
3	0.5	0.1	Fructose+ 0.14g/l $CaCl_2$	1:1	1.5
4	0.5	0.1	Malt extract	1:2	2.0

Fig 3: Shoot tip necrosis during multiplication

Discussion

The present studies have shown that $HgCl_2$ (0.1 percent) was a better sterilant than NaOCl as it resulted in less number of contaminated cultures. Use of various combinations of growth regulators for establishment of peach buds *in vitro* is a common practice and has been well documented (Couto *et al*. 2004; Yeh-jin *et al*. 2007; Casanova *et al*. 2008; Kakani *et al*. 2009) [11-14]. Though a lot of work has been done on serological indexing of *in vitro* raised temperate fruits, there are scanty reports on serological indexing of peach raised *in vitro* (Stein *et al*. 1991; Zilkah *et al*. 2001; Talacko *et al*. 2007; Polak *et al*. 2009; Wang *et al*. 2010; Panattoni *et al*. 2013) [15-20]. Successful shoots regeneration by using different growth regulators and additives for *in vitro* shoot multiplication of peach with nodal segments as explants (Gomez and Segura 1995; Rahman and Bhadra 2011) [21, 22] is well established by using axillary buds as explants for successful micropropagation in peach has also been reported by some workers (Ozaslan *et al*. 2005; Isikalan *et al*. 2010) [23, 24].

Repeated efforts to resolve necrosis under present studies did not succeed thus stressing at further refinement of the culture media to resolve this problem for large scale production of *in vitro* grown peach cv. July Elberta. Shoot tip necrosis has also been reported by Gogoi and Borua (2014) [25] in a review on critical problems encountered in plant *in vitro* culture techniques. *In vitro* multiplication of shoots encountered browning of the shoot tip followed by the basipetal necrosis, senescence and ultimately death of the apical bud. Similar observations have been made by McCown (1985) [26] in woody plants and Arab *et al*. (2016) [27] in G x N 15 hybrid of almond and peach. Shoot tip necrosis in present studies could be a resultant of one or more of many factors such as nutrient deficiency, unbalanced cytokinin level, pH, subculture period, media and its composition and type of gelling agent. Bairu *et al*. (2009) [28] also opined that these factors are crucial in shoot tip necrosis. Besides, deficiency of Ca and B has also been reported to be responsible for shoot tip necrosis (Barghchi and Alderson 1996) [29].

Numerous attempts made for multiplying DAS-ELISA indexed peach cv. July Elberta shoots *in vitro* to obtain PNRSV free plantlets during the course of investigation, could not be succeed as indexed shoots turned necrotic and failed to multiply since shoot tip necrosis could not be controlled even after using additives/absorbents like casein hydrolysate, yeast extract, fructose and malt extract. However, the cause of necrosis could not be ascertained. Therefore, an alternative approach needs to be adopted to overcome the serious problem of shoot tip necrosis observed in the course of studies which appeared to be a major hindrance in the production of PNRSV free *in vitro* shoot regeneration of peach cv. July Elberta.

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