Current Journal of Applied Science and Technology

31(3): 1-9, 2018; Article no.CJAST.45902
ISSN: 2457-1024
(Past name: British Journal of Applied Science & Technology, Past ISSN: 2231-0843, NLM ID: 101664541)

Optimization of Micropropagation from Nodal Segments of Apple (*Malus × domestica. Borkh*) Cultivars Golden Delicious and Red Fuji

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CJAST/2018/45902

Original Research Article

Received 06 November 2018
Accepted 30 November 2018
Published 08 December 2018

ABSTRACT

The present investigation was conducted to develop an effective micropropagation protocol of apple cultivar Golden Delicious and Red Fuji. The nodal segment was used as explants of both the apple (*Malus × domestica. Borkh*) cultivar Golden Delicious and Red Fuji, cultured on Murashige and Skoog's (MS) media supplemented with 35 different combinations of BAP (6-Benzylaminopurine) and NAA (Naphthalene acetic acid). However, MS medium supplemented with 3.33 µM BAP alone was standardized for the shoot cultures of Golden Delicious. On the other hand combinations of 0.05 µM NAA and 4.44 µM BAP was employed for shoot multiplication in Red Fuji. These concentrations supported appreciable length of the micro-shoots. However, highest elongation in both cultivars occurred only when 0.53 µM NAA was used. Moreover, out of the different concentrations of NAA that were tested for Golden Delicious, only 0.53 µM supported highest rooting (average of 7.66 number and length of 22 cm) followed by 0.05 µM NAA where an average of 6.33 root number and 20 cm length were obtained. While Red Fuji showed no root induction in any of the concentrations of NAA tested in the present study. The developed protocols have the potential to be utilized for generation of disease-free, quality planting materials and also in crop improvement programmes.

Keywords: Micropropagation; Golden Delicious; Red Fuji; Murashige and Skoog's; Nodal segment.

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Note: Special issue with selected papers presented in National Conference on Biotechnological Initiatives for Crop Improvement (BICI 2018), December 08-09, 2018, Organized by Bihar Agricultural University, Sabour, Bhagalpur - 813210 (Bihar), India. Conference organizing committee and Guest Editorial Board completed peer-review of this manuscript.

1. INTRODUCTION

Horticultural crops highly influence the economy of many states in the country by supplementing the income and hence call for an increase in quality production to fetch changing market demand. Among temperate horticulture crops, apple (*Malus* × *domestica* Borkh.) is the most important fruit crop of India. Out of various fruit crops, apple is the third most important (64.3 million t/year) in the world [1]. India's apple production area occurs mostly in the North-Western hill regions. The states of Jammu and Kashmir (J&K), Himachal Pradesh (H.P.) and Uttarakhand are main apples producing states of India and to some extent, North-Eastern hills also produce good quality apples. Apple is mainly cultivated through grafting of scion varieties onto clonally propagated rootstocks of a superior variety. Traditional propagation methods do not ensure disease-free and healthy plants, they are season dependent and also results in low multiplication rates. From the mid-20th century, tissue culture has been employed for the propagation of apple through *in vitro* means to explore possibilities of quality planting material as a hit over the conventional method [2]. Although more than 7500 apple varieties have been developed in different countries across the world, only a few of them have qualified as superior varieties. While many varieties are limited by diseases, others do not have the desired quality and hence marketability. Therefore, a major focus of different research programmes has been the development of varieties with economically important traits such as disease tolerance, fruit texture, fruit quality and low-chilling adaptability. Till date, different apple varieties have been produced for varying tastes, flavour, nutritional value (antioxidants, vitamins, and dietary fibres) and suitability to prevailing climatic conditions [3,4,5]. The plants being targeted in the present study Golden Delicious and Red Fuji are commercially important scion varieties and based on productivity both come under the list of present top 10 varieties of the world [6]. Golden delicious is a chance seedling possibly, a hybrid of Grimes Golden and Golden Reinette. It originated from West Virginia in the United States in the 1890's and has been cultivated since then in all the major warm apple growing areas of the world. The variety is a good pollinator. Since Golden Delicious grows easily and bears heavy, light green-yellow coloured fruits having a long shelf life, it is an important variety. However, it is highly susceptible to cedar apple rust and less susceptible to canker, scab, mildew and fire blight. Another selected variety Fuji apple is again a hybrid developed by growers at the Tohoku Research Station in Fujisaki, Aomori, Japan in the late 1930s. Having originated as a cross between the Red Delicious and old Virginia Ralls Genet (Rawls Jennet), the Fuji variety is resistant to scab and mildew and was brought to market in 1962. As compared to other apples, the fruits of this variety have a very long shelf-life requiring no refrigeration. However, with refrigeration, it can remain fresh for up to a year. Its main characteristic include lovely pink speckled flush on yellow-green colored fruits. It is suitable to warm climatic regions but it is highly susceptible to fire blight and less susceptible to cedar apple rust.

The cultivars selected for study, Golden Delicious and Red Fuji have the unique feature of low chilling requirement needs to be propagated in low altitude regions also. Thus, methods for tissue culture and genetic transformation are necessary to improve its shortcoming regarding susceptibility toward various diseases. Therefore the objective of our project is to optimize medium composition for micropropagation. Further, this optimized media can be utilized effectively for micro propagating transformed plantlets in future.

2. MATERIALS AND METHODS

2.1 Plant Material and Establishment of *In vitro* Cultures

Mature trees of apple cultivars Golden Delicious and Red Fuji were selected for the study growing in the Experimental Farm of CSIR-Institute of Himalayan Bioresource Technology, Palampur (32°N, 76°E and 1,230 m above mean sea level) Himachal Pradesh, India. Nodal segments were excised from actively growing shoots of these plants, during May 2014 and used as experimental material. While these explants were used for initiation of shoot cultures after removing all the leaves with the help of a sterile blade, the 2-3 cm long nodal segments were washed for 30 min under running tap water. Thereafter, the explants were washed with liquid detergent (Labolene, Ranbaxy, India) using a stable hair brush and treated with surface fungicide, 50 per cent bavistin (w/v) (BASF India Ltd. India) and 0.5 per cent streptomycin sulphate (w/v) (Sigma, USA) for 10 min. After washing 4-5 times with de-ionized water, the explants were finally surface sterilized under the laminar hood. For

this, they were treated with 70 per cent alcohol (v/v) for 1 min and further with 0.2 per cent mercuric chloride (v/v) containing a drop of Tween-20 as a wetting agent for 5 min. These were rinsed several times with sterile de-ionized water to remove all traces of mercuric chloride. The exposed cut ends were trimmed off with sharp secateurs to eliminate all toxic effects of mercuric chloride. The explants (1.0- 2.0 cm) were then blotted on sterile filter paper and inoculated on basal MS media [7] containing 166.53 µM activated charcoal as polyphenols adsorbent and 567.6 µM ascorbic acid as antioxidant. Sucrose at 3.5 per cent (w/v) and agar at 0.8 per cent (w/v) was invariable, used in the present study. The inoculated explants were then maintained in dark for 45 days under culture room conditions. All aseptic cultures were screened and sub-cultured on fresh medium at 1-week interval until there was no polyphenols exudation. Observations were recorded each day until bud sprouting. Only responsive explants without any fungal and bacterial contamination were screened out.

2.2 Optimization of Medium for Shoot Multiplication of Golden Delicious and Red Fuji

After 45 days, all aseptic cultures obtained as above were transferred to PGR free basal MS media for bud sprouting. When these elongated into 1 cm long young shoots having 5-6 leaves, these were transferred to MS media supplemented with 35 different combinations of BAP and NAA (Table 1). This was done to optimize the medium for shoot growth and multiplication. MS medium without PGRs served as control. While 3.5 per cent sucrose (w/v) was used as a carbon source, 0.8 per cent agar (w/v) served as a gelling agent for the medium. The pH of each medium was maintained at 5.8, and all cultures were incubated under culture lab conditions (i.e., 16 h of cool white fluorescent light having an intensity of 70 $\mu molm^{-2}s^{-1}$ and 8 h dark at 25±2°C). Observations on shoot height and number as well as leaf number were also recorded at 7-day interval for 45 days.

2.3 Statistical Analysis

The data of all experiments were subjected to analysis of variance (ANOVA) and Duncan's multiple tests using STATISTICA data analysis software v7 (STATISTICA release 7, StatSoft Wipro, India). All values were represented as means of treatments ± standard error.

Table 1. Shoot proliferation media composition

Media code	NAA (µM)	BAP (µM)
M1	0	0
M2	0.03	0
M3	0.05	0
M4	0.26	0
M5	0.53	0
M6	0	1.11
M7	0.03	1.11
M8	0.05	1.11
M9	0.26	1.11
M10	0.53	1.11
M11	0	2.22
M12	0.03	2.22
M13	0.05	2.22
M14	0.26	2.22
M15	0.53	2.22
M16	0	3.33
M17	0.03	3.33
M18	0.05	3.33
M19	0.26	3.33
M20	0.53	3.33
M21	0	4.44
M22	0.03	4.44
M23	0.05	4.44
M24	0.26	4.44
M25	0.53	4.44
M26	0	5.55
M27	0.03	5.55
M28	0.05	5.55
M29	0.26	5.55
M30	0.53	5.55
M31	0	6.66
M32	0.03	6.66
M33	0.05	6.66
M34	0.26	6.66
M35	0.53	6.66

3. RESULTS AND DISCUSSION

3.1 Initiation and Screening of Aseptic Cultures

Nodal segments cultured on basal MS medium containing 166.5 µM activated charcoal, 567.7 µM ascorbic acid and 3.5 per cent sucrose showed bud sprouting response after 45-50 days. Various methods have been reported earlier for the control of phenolics induced browning in case of apple. Activated charcoal minimizes explant browning by adsorbing phenolics, inactivating polyphenol oxidases and peroxidises [8,9,10,11]. Another preferred way was the use of antioxidants such as ascorbic acid, citric acid, cysteine and glutathione as they

modify the redox potential of the tissue [8,12]. Fungal and/or bacterial contamination free, aseptic but responsive cultures were then screened out and transferred to fresh medium.

3.2 Optimization of Medium for Shoot Multiplication

In the present study, two varieties were targeted namely Golden Delicious and Red Fuji. Micropropagation has been reported in both varieties namely, Golden Delicious [13,5,14] and Red Fuji [15]. In both these varieties, shoot tips and micro-shoots regenerated from leaves were used as explants, and basal MS medium was invariably used as the culture medium. BAP and NAA were the optimized PGRs in case of Golden Delicious in most of the reports mentioned above, whereas, BAP and IBA were the PGRs of choice in case of Red Fuji [15]. Based on these reports, the effects of different concentrations of NAA (0.03-0.53 µM) and BAP (1.11-6.66 µM) were separately studied on the bud sprouting responses of nodal segments of Golden Delicious and Red Fuji. Interestingly, NAA and BAP supported bud sprouting in both the varieties studied but each variety responded to different concentrations of NAA and BAP. This is not surprising because the success of micropropagation in an apple genotype is dependent on the specific nutrient composition of a culture medium especially plant growth regulators [16] and cannot always be reproduced in another genotype with the same success [17].

3.3 Golden Delicious

In the variety, Golden Delicious, shoot multiplication (as evident from the number of shoots) was highest only when 3.33 µM BAP was used (Fig. 1). This was followed by 1.11 and 2.22 µM BAP. However, browning of explants was observed when the doses were further increased to 5.55 µM and beyond. When both BAP and NAA were used in the culture medium, the number of shoots decreased significantly. This is in opposition to earlier reports where shoots regenerated from leaf explants were multiplied efficiently on medium containing both NAA and BAP [18,14]. In the present study, highest shoot heights were recorded only when 0.26 and 0.53 µM NAA alone were used (Fig. 2). However, when both NAA and BAP were combined, shoot height was invariably and significantly lower, irrespective of concentrations. Rather, the shoot height was almost at par when different concentrations of BAP were combined with that of NAA. In an earlier report, however, PGRs such as GA_3, BAP and NAA had to be combined for higher shoot elongation [14]. Increase in the number of young and unfolded leaves was observed when NAA was combined with BAP. The highest number was, however, recorded in 0.53 µM NAA and 2.22 µM BAP followed by 3.33 µM BAP alone (Fig. 3). The differences in shoot multiplication and shoot elongation responses as observed in earlier reports were probably due to the fact that the initial explants taken were different. While both Mitic et al. [18] and Kumar et al. [14] used shoots regenerated from leaf explants for multiplication and elongation, 1 cm long nodal segments were used in the present study.

All earlier workers have reported the use of IBA for rooting of micropropagated shoots of Golden Delicious [19,20,21,18]. The per cent rooting in these reports varied from 60-97 per cent. As opposed to these reports, however, 100 per cent

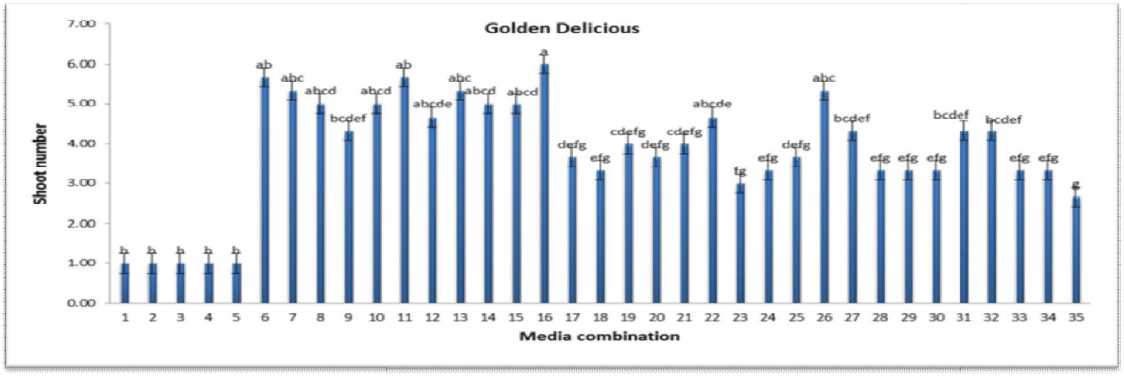

Fig. 1. Effect of different concentrations of BAP and NAA in combination as well as alone on the shoot number in Golden Delicious

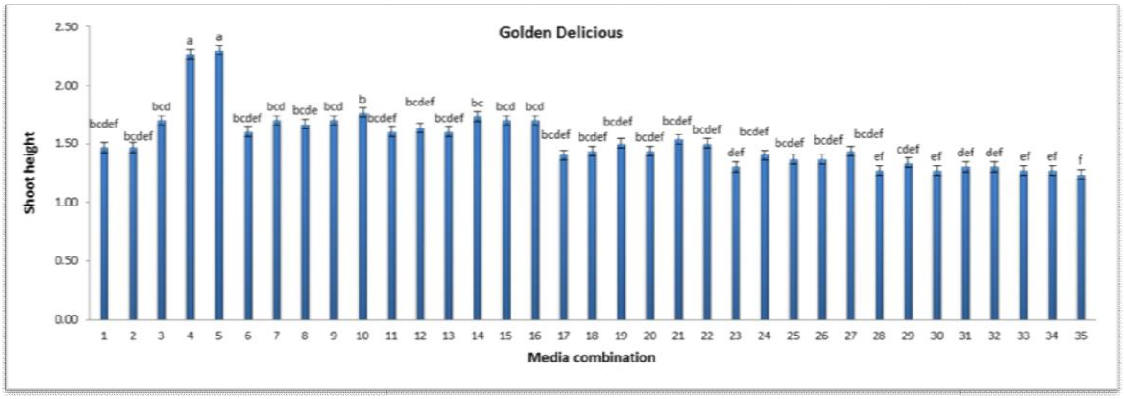

Fig. 2. Effect of different concentrations of BAP and NAA in combination as well as alone on shoot height of Golden Delicious

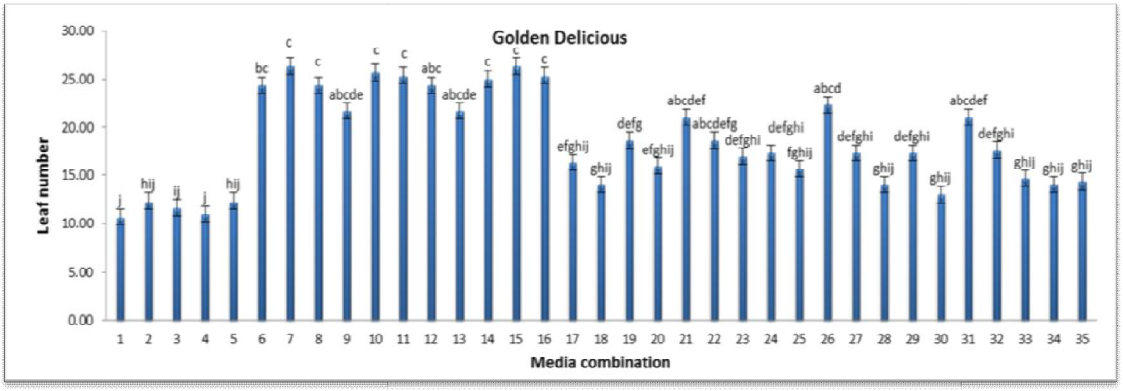

Fig. 3. Effect of different concentrations of BAP and NAA in combination as well as alone on the leaf number in Golden Delicious

rooting was promoted by NAA in the present study. Moreover, out of the different concentrations of NAA that were tested, only 0.53 µM supported highest rooting (average of 7.66 number and length of 22 cm) followed by 0.05 µM NAA where an average of 6.33 root number and 20 cm length were obtained. Finally in the present study, only 3.33 µM BAP was chosen for effective shoot multiplication (Plate 1) of Golden Delicious, whereas, 0.53 µM NAA was selected for effective rooting (Plate 2). Once the nodal segments showing bud sprouting response 35 per cent was transferred to MS basal medium containing 3.33 µM BAP, increase in shoot number was recorded within 7 days. Progressive increase in shoot number, leaf number and also height was recorded with time. Rooting 100 per cent of the micro-shoots occurred within 21 days and the plants became hardened under polyhouse conditions after 60 days.

3.4 Red Fuji

There are only few reports on *in vitro* propagation of Red Fuji. Seong and Song [15] attempted micropropagation of Red Fuji using MS basal medium containing 4.44 µM BAP, 1.5 µM IBA, 3 per cent sucrose, 0.8 per cent agar for shoot multiplication of Red Fuji. However, rooting was achieved only when ½ strength MS medium containing full strength MS vitamins, 1.5 per cent sucrose, 0.8 per cent agar and 1.5 µM IBA were used. In the present study, however, the MS basal medium was supplemented with various concentrations of BAP and NAA (Plate 3). Of these, highest shoot multiplication (Fig. 4) and consequently, the highest number of young and unfolded leaves (Fig. 5) were recorded when 0.05 µM NAA was combined with 4.44 µM BAP. These concentrations supported the appreciable length of the micro-shoots. However, highest elongation occurred only when 0.53 µM NAA

was used (Fig. 6). No root induction was observed in any of the concentrations of NAA tested in the present study. Even when other auxins such as (5.7- 28 µM) IAA and (4.9- 25 µM) IBA were tested, no rooting response was recorded. Rooting was also attempted by Tabori et al. [22]. When the workers used 4.4 to 13 µM BAP, up to 55 per cent decrease in rooting percentage of 'Red Fuji' was recorded as opposed to 51 to 85 per cent increase in presence of 4.4 µM BA and 0.5 µM IBA. Earlier available reports indicated that rooting is possible in Red Fuji, but micro molars of auxin and cytokinins combinations used in the present study are not suitable for rooting. However, effective shoot multiplication was achieved using a combination of NAA and BAP.

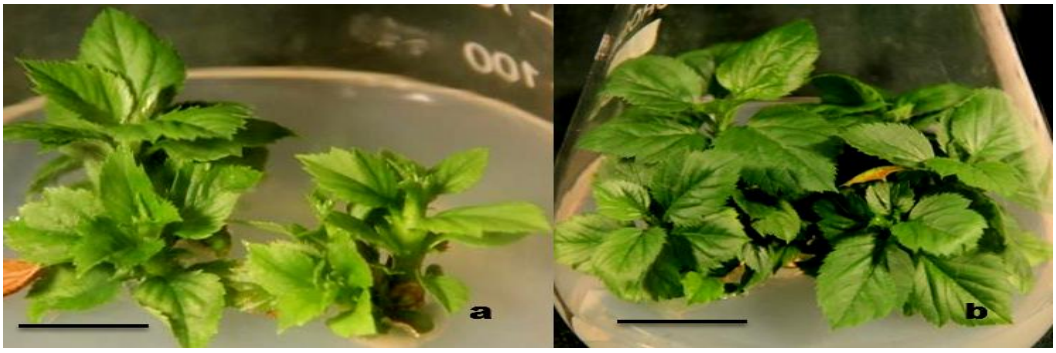

Plate 1. Shoot proliferation in Golden Delicious on MS medium containing only 3.33 µM BAP after (a) 15 days and (b) 45 days of culture

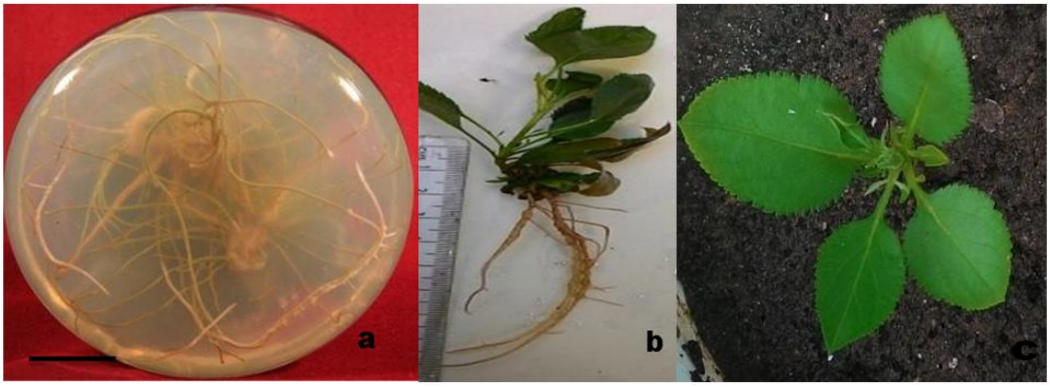

Plate 2. Rooting in Golden Delicious on MS medium containing only 0.53 µM NAA

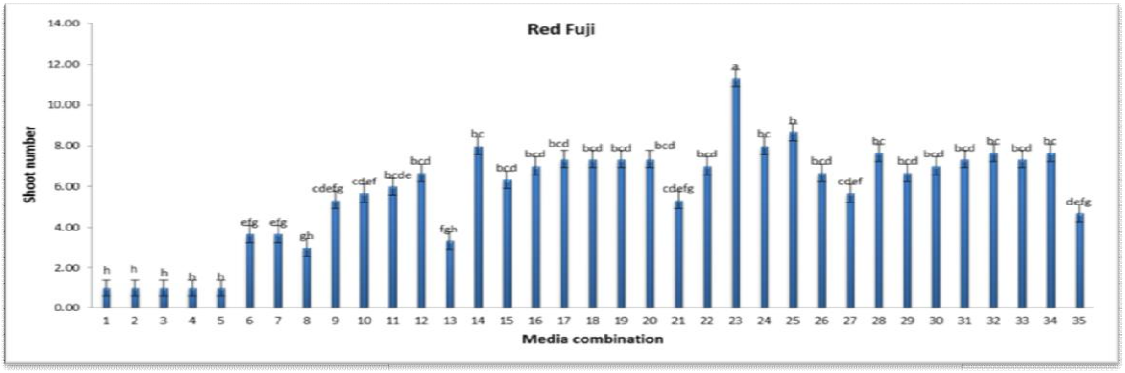

Fig. 4. Effect of different concentrations of BAP and NAA in combination as well as alone on the shoot number in Red Fuji

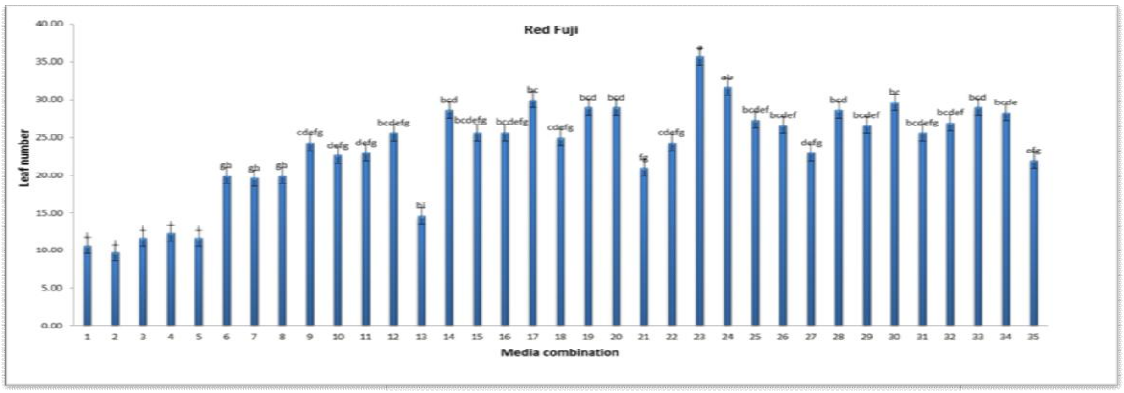

Fig. 5. Effect of different concentrations of BAP and NAA in combination as well as alone on the leaf number in Red Fuji

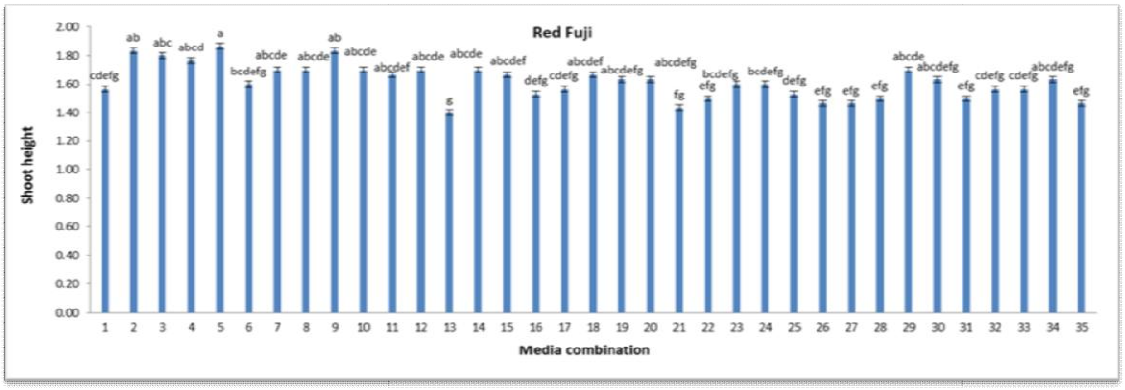

Fig. 6. Effect of different concentrations of BAP and NAA in combination as well as alone on the shoot height in Red Fuji

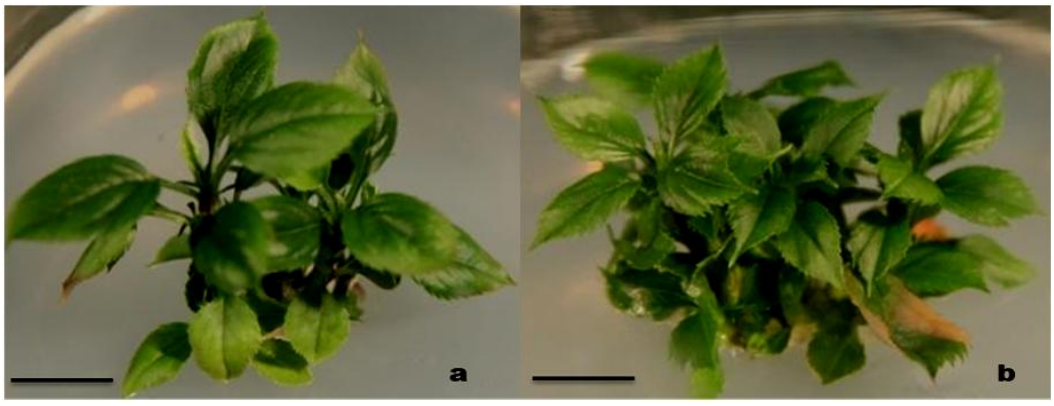

Plate 3. Shoot proliferation in Red Fuji shoots using nodal stem segment from mature trees after (a) 15 and (b) 45 days of culture on MS medium supplemented with 4.44 µM BAP and 0.05 µM NAA

When weekly observed data of both cultivars were included in the statistical analysis, this indicated a strong influence of cultivar and plant growth hormones concentrations on the mean height and number of shoots also leaves number per explant.

4. CONCLUSION

Apple (*Malus* × *domestica* Borkh.) is the fourth most important fruit crop in the world, while India is the fifth largest apple producing country. Commercial cultivation of apple involves grafting of important scion varieties on clonally propagated rootstocks, and there is a large requirement for superior and disease-free planting materials of a particular variety. However major issue is to cope with changing agro-climatic conditions due to a gradual rise in annual mean temperatures has shifted apple cultivation to higher altitudes leading to a significant decline in yield in lower altitudes. In this regard, micropropagation has proven to be an effective method of producing a large number of disease-free planting materials of superior quality and having a low chilling requirement. *In vitro* propagation of two commercially important scion varieties such as Golden Delicious and Red Fuji was, therefore, attempted in the present study. A marked variation in the *in vitro* response of the varieties was observed. The presence of 567.76 µM ascorbic acid and 166.53 µM activated charcoal in 0.8 per cent agar-solidified MS medium containing 3.5 per cent sucrose was crucial for the successful establishment of aseptic cultures in all the studied varieties. However, 3.33 µM BAP was necessary for supporting the highest multiplication of Golden Delicious shoots, while, a combination of both 4.44 µM BAP and 0.05 µM NAA required in case of Red Fuji.

ACKNOWLEDGEMENT

The authors are obliged to Council of Scientific and Industrial Research (CSIR- IHBT), Palampur (H.P.) for providing laboratory facilities to carry out the research work. We are also thankful to the Department of Agriculture Biotechnology, Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur (H.P.).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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