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Regeneration of Plantlets from Cultured Anthers of Tea [*Camellia sinensis* (L.) O. Kuntz.]

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ABSTRACT. *A study was conducted to obtain haploids from cultured anthers of Tea [Camellia sinensis (L.) O. Kuntz.] of clone TRI 2025. Anthers excised from sterilized buds at various lengths were cultured on semisolid media, both in light and dark to select a suitable stage of micro spores which initiate the callus. Subculturing was done once a month. Number of anthers that induced callus was recorded at 2nd month. Anther containing mid-uninucleate micro spores was found to be the most responsive for callus induction. Further studies were carried out using anthers of the selected stage.*

To determine the suitable media for callus formation, anthers were inoculated on seven media with various combinations of auxin and cytokinin and then incubated separately in dark and light. Two subculturings were done at 1st and 2nd month. The weight of callus was measured at the 4th month.

Half Murashige and Skoog (MS) medium (0.4% agar) with 2,4-D (2.0 mg l^{-1}) in combination with kinetin (1.0 mg l^{-1}) or kinetin (1.0 mg l^{-1}) and IAA (1.0 mg l^{-1}) cultured in dark or BAP (1.0 mg l^{-1}) cultured in light were found to be more suitable for callus growth. Yellow and greenish compact calli were obtained in light but whitish calli in dark. Calli formed in these media were transferred to MS medium without and with hormones.

Callusing was observed in all media tested but their growth was not found continuously. Embryoid and meristemoid like structures were observed on the calli formed in dark and light, respectively. However, plant regeneration did not occur during the 5 month period on the shoot growth medium.

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INTRODUCTION

Tea [*Camellia sinensis* (L.) O. Kuntz.], a perennial crop, is cultivated commercially for its tender leaf which is used as a beverage in the world. Due to its economic importance and high demand, scientists are still making attempts to produce elite clones.

The selection of clones from the seedling progenies obtained from hybridization combine high yield potential, leaf quality, pest and disease resistance, *etc.* for commercial plantings. However, self incompatibility, low seed setting ability of high yielding clones (Anandappa *et al.*, 1988), quick loss of viability of seeds and long durations of each sexual cycle have been stumbling blocks in tea breeding programmes.

Tissue culture techniques have a wide application in breeding programmes. The tissue vulture techniques shorten time taken to obtain and select new clones with interesting characteristics and also increase the production of tea in order to meet its ever increasing demand. Improvement of tea using cellular and molecular biology techniques is difficult, because cell culture procedures are not well established (Kato, 1996). However, anther culture technique has the great potential in tea improvement programmes by rapid production of homozygous lines from heterozygous breeding lines, getting gametaclonal variations and early expression of recessive genes. By doubling their chromosome number, homozygous diploids can be produced in a much shorter time. It would, therefore be useful for research in tea breeding programmes for further improvement of both yield and quality. In tea, anther culture was first described by Katsuo (1969) however, very low interest has been shown by the scientists on this aspect. Hence, an attempt was made to regenerate plantlets from cultured anthers of a Sri Lankan tea clone.

MATERIALS AND METHODS

Experiment 1

This experiment was done to select a suitable stage of micro spores in tea anthers to produce the callus. Unopened floral buds in successive development stages of clone TRI 2025 were harvested and separated into 6 groups depending on the morphological complexity of the buds. Length of individual floral bud from each group was measured and the colour of the anthers was observed. The development stages of micro spores within the

anthers from each bud group were identified by microscopic observation of stained pollen as reported by Li *et al.* (1983) in wheat anther culture.

After separating the harvested buds, they were stored in a refrigerator at 5°C for 3 days. They were then sterilized with 70% ethanol for 1 min and 5% Clorox (Sodium Hypochlorite 5.25%) for 10 min. The anthers excised separately from the buds in successive morphological stages were inoculated on Murashige and Skoog (MS) medium (0.4% agar) supplemented with 2,4-D (6.0 mg l^{-1}) alone or 2,4-D (6.0 mg l^{-1}) and Kinetin (2.0 mg l^{-1}). For each treatment, four Petri dishes (15 × 90 mm) each containing 15–20 anthers were incubated separately in light (2000 lx) and dark. Subculturing was done once a month. In each treatment, number of anthers that induced calli were recorded at 2nd month.

Experiment 2

As the results of Experiment 1 showed that anthers containing micro spores at mid-uninucleate stage were the most responsive to produce the callus, further studies were carried out using anthers of the selected stage.

To determine a suitable medium for callus formation, anthers excised from sterilized buds were inoculated on half MS medium (0.4% agar) supplemented with 2,4-D (2.0 mg l^{-1}) alone or in combination with BAP (1.0 mg l^{-1}) or Kinetin (1.0 mg·l^{-1}) or Kinetin (1.0 mg l^{-1}) and IAA (1.0 mg l^{-1}) and on MS medium (0.4% agar) supplemented with 2,4-D (6.0 mg l^{-1}) alone or in combination with either BAP (2.0 mg l^{-1}) or Kinetin (2.0 mg l^{-1}). Fifteen anthers were placed in each Petri dish and were incubated separately in light (2000 lx) and dark. Two subculturings were done at 1st and 2nd month from culture initiation. In each treatment, number of anthers that induced callus was recorded at 2nd month. Fresh weight of callus formed from anthers was measured at 4th month and repeated 5 times. The data on weight of callus was analysed using Analysis of Variance (ANOVA). Means were compared using Duncan's multiple range test (Gomez and Gomez, 1984). Colour and type of callus were also observed.

The calli formed in half MS medium supplemented with 2,4-D (2.0 mg l^{-1}) in combination with BAP (1.0 mg l^{-1}) and cultured in light or Kinetin (1.0 mg l^{-1}) or Kinetin (1.0 mg l^{-1}) and IAA (1.0 mg l^{-1}) cultured in dark were transferred to MS medium (0.6% agar) supplemented with IAA (3.0 mg l^{-1}) and BAP (1.0 mg l^{-1}) or BAP (3.0 mg l^{-1}) and GA3 (1.0 mg l^{-1}) or BAP (3.0 mg l^{-1}) to induce organogenesis or embryogenesis. The cultures were

incubated in light (3000 lx). Calli were transferred to fresh media once a month until regeneration of shoot buds was observed.

RESULTS AND DISCUSSION

Experiment 1

Cytological analysis of micro spores within the anther of each bud group revealed that most of the micro spores in white colour anther excised from a bud of 3 mm in length contained nuclei only (early–uninucleate stage). The greenish-yellow to shining yellow coloured anthers isolated from buds of 4-5 mm in length, which contained mocrospores with a centrally located nucleus lying in a thin layer of peripheral cytoplasm, were at mid-uninucleate stage. The dark yellow coloured anthers from buds of 6 mm in length, containing micro spores with peripheral nucleus, were at the late-uninucleate stage. Anthers isolated from buds more than or equal to 7 mm in length had binucleate pollen. Since the stage of micro spores is a critical factor for *in vitro* androgenesis, it is important to use the bud of the correct stage.

The results indicated that the % of callus induction from anthers excised from 5 mm bud length was relatively high (up to 32%) as shown in Figure 1. In this stage, anthers contained micro spores at mid–uninucleate stage. The anthers isolated from buds of less than 8 mm length induced callus. Callus was first initiated in anthers obtained from buds of 5 mm in length that were grown in MS medium with 2,4-D and kinetin. Yellow, compact calli were obtained in both dark and light culture conditions.

Most of the calli induced at the early uninucleate stage were derived from anther wall and connective tissues. However, anther lobes were swollen and became whitish in other stages. Then they burst and produced callus. Calli induced from connective tissues normally did not grow rapidly. Anther containing binucleate pollen was not responsive for callus induction. They turned brown and died during first 2–4 weeks.

The optimum stage of pollen development at the time of harvest of the buds is of vital importance but differs between species. Several researchers have suggested that the most productive anthers contain uninucleate micro spores midway between release from the tetrad and the first pollen grain mitosis (Sunderland, 1974; Niizeki, 1977; Sunderland and Dunwell, 1977; Zhu *et al.*, 1980). Sunderland (1982) summarized that anthers of tobacco give the best response if pollen was cultured at first mitotic or later

stage. Sun (1978) reported that when rice anthers were inoculated at the binucleate stage, most pollen grains died.

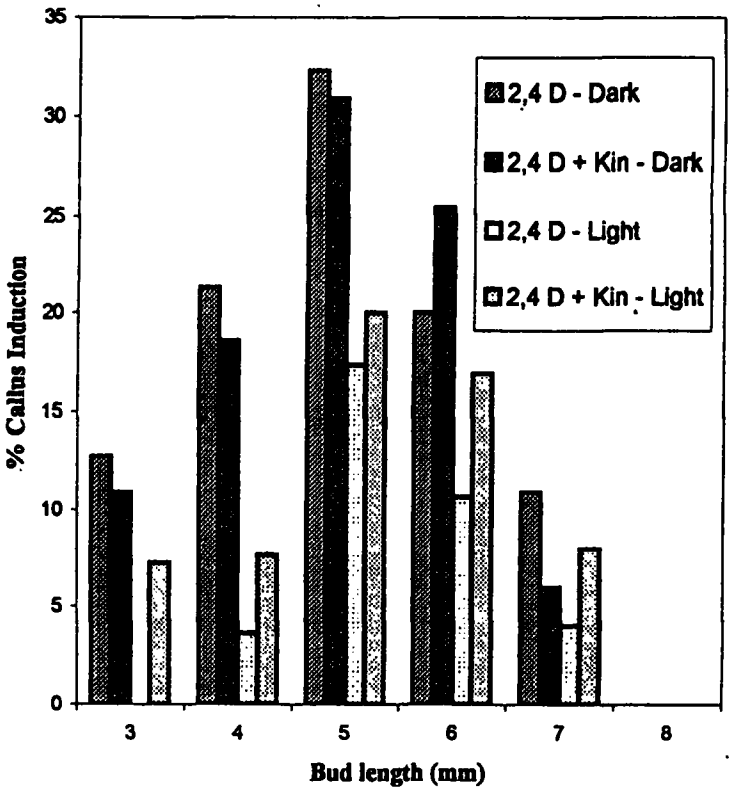

Figure 1. The percentage of callus induction in anthers obtained from buds at different lengths.

After 5 months of culture, initiation of root was observed in peripheral region of callus. Similar results were described by Doi (1981) in tea anther culture. This may be due to the high concentration of 2,4-D (6.0 mg l^{-1}) presented in MS (agar 0.4%) medium. Cellular structure was identified in roots by microscopic observation.

Experiment 2

The induction of calli varied between half MS and MS media grown in light and dark (Figure 2). A high rate of callus induction was obtained in half MS medium with 2,4-D (2.0 mg l^{-1}) and kinetin (1.0 mg l^{-1}) incubated in dark (60%) and 2,4-D (2.0 mg l^{-1}) and BAP (1.0 mg l^{-1}) grown in light (53%). Chen (1984) described that the decrease of NO_3^- ion concentration in MS medium is favourable for the development of pollen embryos from rubber anthers.

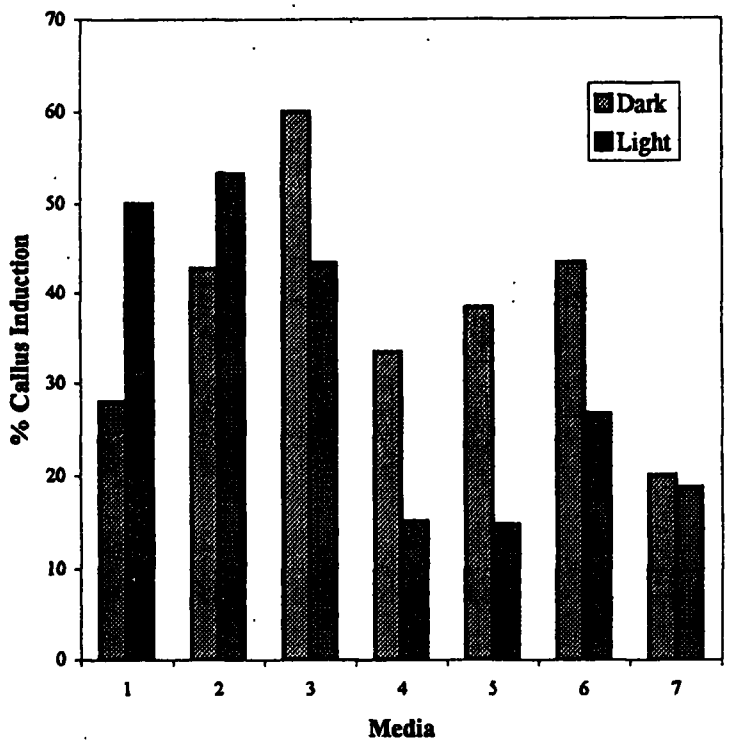

Figure 2. The percentage of callus induction in anthers cultured on different media.

[Note: 1- ½ MS+2,4-D (2 mg l^{-1}); 2 - ½ MS+2,4-D (2mg l^{-1}) + BAP (1 mg l^{-1} 3- ½ MS+ 2,4-D (2 mg l^{-1}) + Kinetin (1 mg l^{-1}); 4- ½ MS + 2,4-D (2 mg l^{-1}) + Kinetin (1 mg l^{-1})+IAA (1 mg l^{-1}); 5- MS+2,4-D (6 mg l^{-1}); 6- MS+2,4-D (6 mg l^{-1})+Kinetin (2 mg l^{-1}); 7- MS+2,4-D (6 mg l^{-1})+BAP (2 mg l^{-1})]

The browning and blackening of the callus were more in MS medium when compared to those found in half MS medium. High concentration of inorganic salts and growth regulators may cause this phenomenon. This observation confirm with those of Sarwar (1985) who reported that inorganic salts of MS medium at low concentrations reduced the explant browning. Beneficial effect of low salt concentration was also described in Azaleas (Anderson, 1975; Ma and Wang, 1977; Preil and Engelhardt, 1977).

The mean weight of callus varied significantly ($p<0.001$) between 7 media (Figure 3). The callus growth was generally more in half MS media with various combinations of auxin and cytokinin than in MS media,. Although the callus initiated in MS media, the growth was unsatisfactory. The results of the present study are in agreement with those reported by several researchers Chu (1978) demonstrated that a low nitrogen concentration (490 mg l^{-1}) was beneficial for callus production from rice anthers whereas a higher

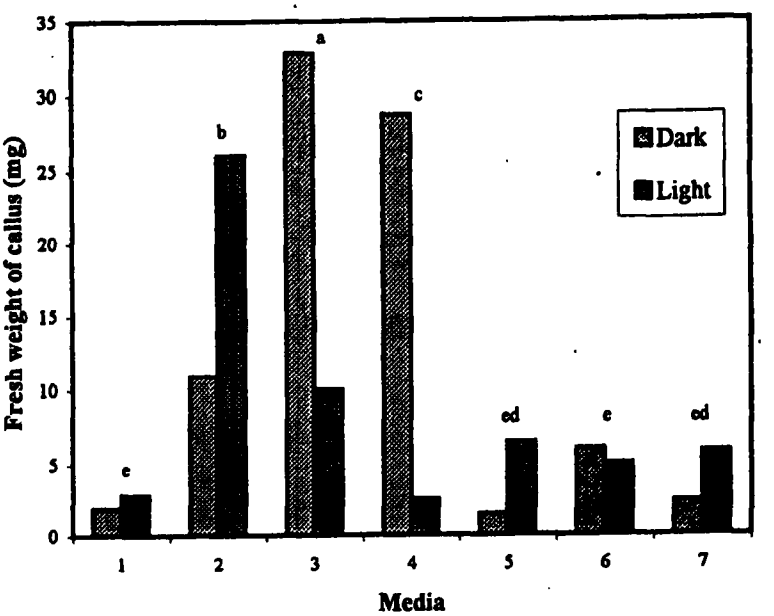

Figure 3. Response of media on growth of callus from anthers.
[Note: 1- ½ MS+2,4-D (2 mg l^{-1}); 2 - ½ MS+2,4-D (2mg l^{-1})+BAP (1 mg l^{-1}) 3 - ½ MS + 2,4-D (2 mg l^{-1}) + Kinetin (1 mg l^{-1}); 4- ½ MS + 2 ,4-D (2 mg l^{-1}) +Kinetin (1 mg l^{-1})+IAA (1 mg l^{-1}); 5- MS+2,4-D (6 mg l^{-1}); 6- MS+2,4-D (6 mg l^{-1})+Kinetin (2 mg l^{-1}); 7- MS+2,4-D (6 mg l^{-1})+BAP (2 mg l^{-1}); means with the same letter are not significantly different at p = 0.05].

concentration was inhibitory. The salt mixtures for the anther culture in Solanaceae have been reported to be half strength Murashige and Skoog (1962) mixture (Dunwell, 1986).

Of the seven media tested, three treatments with the half MS medium were found to be more suitable for callus formation. Medium with 2,4-D (2.0 mg l^{-1}) and either Kinetin (1.0 mg l^{-1}) or Kinetin (1.0 mg l^{-1}) and IAA (1.0 mg l^{-1}) were effective in dark, whereas medium with 2,4-D (2.0 mg l^{-1}) and BAP (1.0 mg l^{-1}) the was best in light. Most of anthers formed yellow firm calli in MS media. In half MS media, yellow and greenish compact calli were obtained in light but whitish calli in dark culture condition. Skoog and Miller (1957) reported that auxin and cytokinin interact *in vitro* to regulate cell division and differentiation.

After transferring the calli to differentiation media, callusing was observed in all media tested but their growth was not found continuously. Embryoid like structures were observed on the calli formed in dark but, in most cases, they changed to brown colour and died. The early death of microspore derived embryos has also been reported for other woody species (Milewska–Pawliczuk and Kubicki, 1977; Milewska–Pawliczuk, 1990; Zhang *et al.*, 1990). Radojevic (1991) described that auxin (2,4-D) and kinetin were necessary factors for the induction of both androgenesis and somatic embryogenesis while for the further development of androgenic and somatic embryos, auxin (IAA or IBA) and GA_3 were required.

When calli formed in light were grown in MS medium with BAP alone or in combination with GA_3, they turned into dark green, meristemoid like structures. However, plant regeneration did not occur during 5 months period on shoot growth medium. Doi (1981) was able to produce callus from anther but was unable to regenerate shoots. In contrast, Zhenguang and Huihuo (1987) described a technique through which plantlets were differentiated from anther callus.

CONCLUSIONS

The present study shows that the exact stage of micro spores can be determined by a cytological examination. However, for large scale programmes, bud length and colour of anthers could be used as parameters to select the correct stage of micro spores for good callus production. The micro spores at mid-uninucleate stage appears to be the most responsive for callus

induction from anthers excised from buds of 5 mm in length. However, mid to late-uninucleate stage would also be effective.

Of the seven media tested, half MS basal medium is more suitable for callus formation. Half MS with 2,4-D (2.0 mg l^{-1}) and either kinetin (1.0 mg l^{-1}) or kinetin (1.0 mg l^{-1}) and IAA (1.0 mg l^{-1}) are effective in dark but medium with 2,4-D (2.0 mg l^{-1}) and BAP (1.0 mg l^{-1}) is also effective in light .

In the present study of haploidization, embryoid and meristemoid like structures were observed on the calli formed in dark and light, respectively. However, plantlets could not be regenerated yet. Therefore, continued effort and screening of calli with regenerative capacity is required to produce the haploids.

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