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# Callus Induction and *in vitro* Complete Plant Regeneration of Different Cultivars of Tobacco (*Nicotiana tabacum* L.) on Media of Different Hormonal Concentrations

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**Abstract:** In the present study, regeneration conditions for two-cultivars-of-tobacco (*Nicotiana tabacum* L.) were optimized. At different concentrations the effects of 1-naphthalene acetic acid (auxin)-and-6-benzylaminopurine (cytokinin)-on-callus-induction-and-subsequent-plant regeneration in K-399 and SPTG-172 using Murashige and Skoog (MS) media were studied. The seeds were germinated on simple MS media, while MS media with auxine and cytokinin was used for callus induction and regeneration of shoots. In tobacco, callus was successfully induced from leaf explants using a high ratio of auxin: Cytokinin. The over all callus induction frequency in SPTG-172 was greater than K-399 indicating a difference in the genetic background of two cultivars. Both cultivars also showed best response to different hormonal concentrations. The resultant calli were stimulated to form shoots by increasing the cytokinin concentration and decreasing auxin content of culture media. There were clear differences between the two cultivars in their ability to produce regenerated plants. The maximum number of calli were regenerated in SPTG-172 whereas the mean number of shoots produced per callus at all concentrations was higher in K-399 indicating that's shoot regeneration is markedly affected by the genotype. The roots developed well on hormones free MS media in both cultivars irrespectively of the varietal difference. The well-rooted plants were then transferred to green house for acclimatization-to-the *in vitro* environment.

**Key words:** *Nicotiana tabacum*, callus induction, plant regeneration 1-naphthalene acetic acid, 6-benzylaminopurine

## INTRODUCTION

Tissue culture is the process whereby small pieces of living tissue (explants) are isolated from an organism and grown aseptically for indefinite periods on a nutrient medium under controlled conditions. *In vitro* cultivation of plants is a necessary step in a large amount of experiments: Micropropagation, creation of virus free plants, genetic transformation, etc. (Georgieva *et al*., 1996). The most common result obtained *in vitro* is the proliferation of the explant into a mass of relatively undifferentiated tissue called callus which is an amorphous mass of loosely arranged thin walled parenchyma cells developing from proliferating cells of the parent tissue (Dodds and Robert, 1985). Callus from an explant tissue *in vitro* is formed as a result of dramatic changes in the appearance and metabolism of the cells (Aitchison *et al*., 1978). Growth regulator concentrations in culture medium are critical for the control of growth and morphogenesis. The phytohormones, cytokinin and auxin are known to promote callus formation in tissue culture (Skoog and Armstrong, 1970; Letham, 1974; Akiyoshi *et al*., 1983). Auxin effect plant growth and morphogenesis e.g., Indole-3-Acetic Acid (IAA), a natural auxin of higher plants, is involved in regulating cell elongation, cell division and differentiation (Dietz *et al*., 1990). Cytokinins can promote cell enlargement in certain tissues (Rayle *et al*., 1982; Ross and Rayle, 1982). The balance between auxins and cytokinins controls the formation of roots, shoots and callus tissue *in vitro* (Skoog and Miller, 1957), the outgrowth of shoot axillary buds (Cline, 1994, 1996; Tamas, 1995) and the formation of lateral roots (Wightman *et al*., 1980). During the past few decades tissue culture techniques have been developed that could be used for the improvement of plants. Callus culture is one of the most important plant science techniques for developing clonal populations, plant regeneration and genetic manipulation in both monocotyledon and dicotyledonous plants (Reinert and Bajaj, 1976). The callus culture of tobacco, a dicotyledonous plant has proved extremely useful for studying the cellular basis of morphogenesis and has enabled the complex influence of hormones on this process. The aim of this research was to study the effect

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of, 6-benzylaminopurine (Cytokinin) and 1-naphthalene acetic acid (Auxin) at different concentrations on callus induction and shoots regeneration rate from *Nicotiana tabacum* L. cultivars to optimize tissue culture conditions.

## MATERIALS AND METHODS

Experimental studies were conducted on *Nicotiana tabacum* L. var. SPTG-172 and K-399 taken from Pakistan Tobacco Company, Buneer. This research work was conducted in department of biotechnology, university of Malakand in 2006. Tobacco seeds after soaking in water for 24 h were surface sterilized by immersion in 70% ethyl alcohol for two minutes and 0.1% mercuric chloride for 5 min (Ekrum, 2001). Before culturing, the seeds were washed three times with sterilized distill water. The seeds were germinated on agar media containing 3.0% sucrose and 0.8% bacto agar (Difco), the basal medium used was of Murashige and Skoog (1962) (MS) salt solution with out hormones. Leaf segments from tobacco seedlings developed on MS media after one month were used for callogenesis. Callus induction and shoots regeneration from callus was obtained by using MS medium modified with 1-naphthalene acetic acid (auxin) and 6-benzylaminopurine (cytokinin). The callus was induced from leaf explant on media contained MS (Murashige and Skoog, 1962) salts, vitamins, 30 g $L^{-1}$ sucrose, 8 g $L^{-1}$ bacto-agar (Difco), 6-benzylaminopurine (0.2 mg $L^{-1}$) and 1-naphthalene acetic acid (0.0, 1.0, 2.0 or 3.0 mg $L^{-1}$). The shoots were regenerated from callus on media containing MS (Murashige and Skoog, 1962) salts, vitamins, 30 g $L^{-1}$ sucrose, 8 g $L^{-1}$ bacto-agar (Difco), 6-benzylaminopurine (0.0, 1.0, 2.0 or 3.0 mg $L^{-1}$) and 1-naphthalene acetic acid (0.2 mg $L^{-1}$). The pH was adjusted during each experiment to 5.8 with 1 N NaOH or 1 N HC1 using electronic pH indicator. All the operations and inoculations were carried out under aseptic conditions in laminar airflow cabinet. The medium was heated to dissolve solutes and then dispensed in test tubes for seeds germination and in flasks for callus induction and shoots regeneration and autoclaved at a temperature of 121°C at a pressure of 151 bs psi for 20 min and cooled at room temperature (Puchooa *et al*., 1999). The cultures were kept in controlled environment at 25°C and 16-8 h light\dark regime under fluorescent light.

## RESULTS

The cultivar SPTG-172 produced calli on all the combination of growth regulators in 4 weeks at 25°C and 16-8 h light\dark regime under fluorescent light. However, maximum callus induction in term of callus size, callus weight and percentage of calli produced from explants were observed at 0.2 mg $L^{-1}$ benzyl aminopurine and 2.0 mg $L^{-1}$ 1-naphthalene acetic acid (MS + 0.2 mg $L^{-1}$ BAP + 2.0 mg $L^{-1}$ NAA) followed by variable callus induction response on the other combination used as shown in Table 1. The percentage of calli produced at this concentration was 67.85% with a mean size and mean weight of 1.97 cm and 2.08 g, respectively. In addition, the calli produced were compact in texture and greenish in color (Fig. 1). The cultivar K-399 produced calli on all the growth regulators-combination-except 0.0 mg $L^{-1}$ NAA and 0.2 mg $L^{-1}$ benzylaminopurine (MS + 0.0 mg $L^{-1}$ NAA + 0.2 mg $L^{-1}$ BAP) in 4 weeks at 25°C and 16-8 h light/dark regime under fluorescent light. The most favorable combination of growth regulators at which the K-399 cultivar showed excellent callus induction was 3.0 mg $L^{-1}$ naphthalene acetic acid and 0.2 mg $L^{-1}$ benzyl aminopurine (MS + 3.0 mg $L^{-1}$ NAA + 0.2 mg $L^{-1}$ BAP). The percent calli produced at this combination of growth regulators was 59% with a mean size and mean weight of 1.75 cm and 1.81 g, respectively as shown in Table 1. In addition, the calli were soft in texture and greenish in color (Fig. 2).

Shoots formation from calli of SPTG-172 and K-399 were successfully obtained on different concentrations of

Fig. 1: Callus in SPTG-172 after 4 weeks

Fig. 2: Callus in K-399 after 4 weeks

Table 1: The percent calli formation, mean size, mean weight of cultivar SPTG-172 and K-399 after 4 weeks

| Concentration | Cultivar SPTG-172 | | | | Cultivar K-399 | | | |
|---|---|---|---|---|---|---|---|---|
| NAA:BAP | Calli (%) | Mean size (cm) | Mean weight (g) | Callus texture | Calli (%) | Mean size (cm) | Mean weight (g) | Callus texture |
| 0.0:0.2 | 15.00 | 0.65 | 0.53 | Soft and yellowish | - | - | - | - |
| 1.0:0.2 | 34.00 | 1.58 | 1.43 | Hard and light green | 33 | 1.34 | 1.40 | Compact and light green |
| 2.0:0.2 | 7.85 | 1.97 | 2.08 | Compact and greenish | 46 | 1.66 | 1.65 | Soft and greenish |
| 3.0:0.2 | 0.76 | 1.47 | 1.07 | Soft and light green | 59 | 1.75 | 1.81 | Soft and light green |

NAA (1-naphthalene acetic acid); BAP (6-benzylaminopurine)

Table 2: The percent calli regenerated and number of shoots per callus in SPTG-172 and K-399 cultivars after two and four weeks

| Concentrations | Cultivar SPTG-172 | | | | Cultivar K-399 | | | |
|---|---|---|---|---|---|---|---|---|
| BAP: NAA | Calli regenerated after 2 weeks (%) | No. of shoots/ callus after 2 weeks | Calli regenerated after 4 weeks (%) | No. of shoots/ callus after 4 week | Calli regenerated after 2 weeks (%) | No. of shoots/ callus after 2 weeks | Calli regenerated after 4 weeks (%) | No. of shoots/ callus after 4 week |
| 0.0:0.2 | - | - | - | - | - | - | - | - |
| 1.0:0.2 | 20 | 1.57 | 25.00 | 2.00 | 32 | 2.14 | 36 | 2.50 |
| 2.0:0.2 | 41 | 2.07 | 44.12 | 2.34 | 15 | 2.00 | 15 | 2.60 |
| 3.0:0.2 | 26 | 1.71 | 29.60 | 1.87 | 22 | 1.80 | 25 | 1.90 |

NAA (1-naphthalene acetic acid); BAP (6-benzylaminopurine)

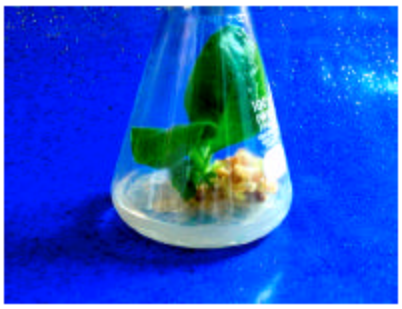

Fig. 3: Shoots regeneration from callus in SPTG-172 after 4 weeks

Fig. 5: Rooting in SPTG-172 on hormones free media

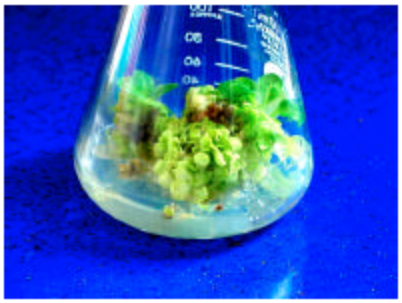

Fig. 4: Shoots regeneration from callus in K-399 after 4 weeks

Fig. 6: Rooting in K-399 on hormones free media

BAP and NAA in MS medium at 25°C and 16-8 h light\dark regime under fluorescent light except on medium containing 0.2 mg $L^{-1}$ NAA and 0.0 mg $L^{-1}$ BAP where no regeneration was observed for both cultivars. The combination of 2.0 mg $L^{-1}$ BAP and 0.2 mg $L^{-1}$ NAA (MS + 2.0 mg $L^{-1}$ BAP + 0.2 mg $L^{-1}$ NAA) were found the most favorable in term of percent calli regenerated and number of shoots per callus for SPTG-172 (Fig. 3 and Table 2). The percentage of calli forming shoot at this concentration was 41% and the mean number of shoots per callus was 2.07 at 14th day. The improvement in shoots forming ability of calli and mean number of shoot per callus at 28th day was found 3.12% and 0.27, respectively as shown in Table 2. The calli of cultivar K-399 showed best response to combination, 1.0 mg $L^{-1}$ BAP and 0.2 mg $L^{-1}$ NAA (MS + 1.0 mg $L^{-1}$ BAP + 0.2 mg $L^{-1}$ NAA) however, the percentage of calli

regenerated were lower then SPTG-172. The regenerated calli percentage was 32% with a mean of 2.14 shoots per callus at 14th day of inoculation and an improvement of 4% in the percentage of regenerated calli with a mean increase of 0.36 shoots per callus was observed at 28th day (Table 2 and Fig. 4). Adventitious roots appeared in both cultivars within 2 weeks on hormone-free MS media and developed further in 4 weeks irrespectively of the varietal difference (Fig. 5 and 6). The roots developing ability of SPTG-172 and K-399 was similar on hormones free MS media. The complete regenerated plants of both cultivars were then transferred to green house.

$$\text{Callus induction frequency} = \frac{\text{No. of calli produced}}{\text{Total No. of explant}} \times 100$$

$$\text{Shoot induction frequency} = \frac{\text{No. of calli forming shoots}}{\text{Total No. of calli}} \times 100$$

## DISCUSSION

Laboratory experiments were conducted to optimize the conditions for callus induction and regeneration of *Nicotiana tabacum* plant cultivars. The experiments were carried under aseptic conditions and no contamination was observed in each culture, no rot or mold were observed around the seedlings and aseptic conditions were maintained throughout the experiments. The seeds of *Nicotiana tabacum* were germinated on plane agar and MS media with out any growth regulators (Vissenberg *et al*., 2001). The seeds germination was best on Murashige and Skoog (1962) media mainly due to the availability of nutrients in sufficient amount as compared to plane agar media consisting of only 3% sucrose, 0.8% agar and distilled water. Temperature around 25°C and photoperiod of 16 h light and 8 h dark were favourable for germination and further growth of seedlings (Ekrum, 2001). The seed germination in the presence of light confirmed the result of Bradbeer (1988) reported that light is a requirement for germination of tobacco (*N. tabacum* L.) seeds. Calli were induced in four weeks by taking leaf explants from *in vitro* grown plants on MS media supplemented with various concentrations of cytokinins and auxins. Callus initiation was observed after one week of culture in all the combinations tested, irrespective of varietal and media differences except 0.0 mg $L^{-1}$ NAA + 0.2 mg $L^{-1}$ BAP, at which K-399 showed no callogenesis. The callus production from leaf explants of tobacco *in vitro* could be influenced by the hormonal concentration in medium and genotype of cultivar. Keeping in view the importance of media recipe and response of genotype on callus induction, this study was proposed to evaluate the varietal response of tobacco cultivars (K-399 and SPTG-172) to callus induction and regeneration. In tobacco, high concentration of auxin-(1-naphthalene acetic acid) and low concentration of cytokinin (6-benzylaminopurine) was found better for callogenesis in both cultivars and was in contrast to Ekrum (2001) reported a high concentration of 6-benzylaminopurine (1.0 mg $L^{-1}$) and low concentration of 1-Naphthalene acetic acid (0.1 mg $L^{-1}$) for callus induction in tobacco. Colak *et al*. (1998) used 1 mg $L^{-1}$ Indole-3-acetic acid and 2 mg $L^{-1}$ Kinetin for inducing callus in the anther cultures of *Nicotiana rustica* L. It might be mainly due to the use of different cultivars and different explants for callus induction in the present study. The differences in the response of tobacco cultivars to different hormonal concentrations were noted in term of percentage calli formed, mean size and mean weight of calli. The statistical analysis showed that most suitable concentration for SPTG-172 was 2.0 mg $L^{-1}$ NAA + 0.2 mg $L^{-1}$ BAP confirming the results of Fajkus *et al*. (1998) and Fojtova *et al*. (2003). The tobacco cultivar K-399 showed best response to hormonal concentration 3.0 mg $L^{-1}$ NAA + 0.2 mg $L^{-1}$ BAP and was different from the result Fajkus *et al*. (1998) mainly due to varietal difference. The percent calli produced, mean size and mean weight of calli was greater in SPTG-172 at 2.0 mg $L^{-1}$ NAA + 0.2 mg $L^{-1}$ BAP then K-399 at 3.0 mg $L^{-1}$ NAA + 0.2 mg $L^{-1}$ BAP. The best response to different hormonal concentration was probably due to any little differences in the genetic background of tobacco cultivars used in this investigation. The shoots appeared in both cultivars on modified MS media containing a low concentration of 1-naphthalene acetic acid and high concentration of 6-benzylaminopurine and confirmed the result of Skoog and Miller (1957) demonstrated that a low ratio of auxin: Cytokinin stimulated the formation of shoots in tobacco callus. The results obtained were different from Dhaliwal *et al*. (2004) reported that Shoot Formation (SF) medium required only exogenous cytokinin ($N^6$-benzyladenine) for *N. tabacum* L. Shoot regeneration was markedly affected by the genotype of explant in that different cultivars of a given species show quite different frequencies of shoot regeneration. The average shoot induction frequency in K-399 was greater then the shoot induction frequency of SPTG-172 where as the percentage of calli regenerated in K-399 was lower then SPTG-172. The hormonal concentration at which SPTG-172 produced maximum number of shoots was 2.0 mg $L^{-1}$ BAP + 0.2 mg $L^{-1}$ NAA which correlate the findings of Fajkus *et al*. (1998). The cultivar K-399 showed

maximum number of shoot at hormonal concentration of 1.0 mg $L^{-1}$ BAP + 0.2 mg $L^{-1}$ NAA and this was in accordance to the result of Georgieva *et al.* (1996) reported that nutritional MS medium containing high cytokinin (1.0 mg $L^{-1}$ BAP) and low auxin (0.1 mg $L^{-1}$ NAA) is a well-established one for regeneration of tobacco tissue. The difference in regeneration ability of tobacco cultivars might be due to hormonal concentration in medium, endogenous hormones content of explants and differences in the genotype of both cultivars used in this study. The results also showed that there was a minute increase in the shoots forming ability of both cultivars during the 3rd and 4th week and most of the shooting occurred in 1-2 weeks of culture. This was similar to Thorpe and Murashige (1970) who reported that the earliest histological events leading to shoot formation in tobacco callus occur between the 6th and 14th days of culture. This was in accordance to earlier research of Skoog and Miller (1957). The shoots regenerated from callus were transferred to roots induction media. In both cultivars rooting occurred well on hormones free media with in 1-2 weeks irrespectively of varietal difference. The same result was obtained by Puchooa *et al.* (1999) reported that roots formation occurred just after shoot regeneration and exogenous growth regulators are not required. The rooting in regenerated shoots on hormones free media was also reported by Fojtova *et al.* (2003). It can be concluded from present study that cultivar SPTG-172 showed maximum callus induction at 0.2 mg $L^{-1}$ benzyl aminopurine and 2.0 mg $L^{-1}$ naphthalene acetic acid while the most favorable combination of growth regulators at which K-399 cultivar showed an excellent callus induction was 3.0 mg $L^{-1}$ naphthalene acetic acid and 0.2 mg $L^{-1}$ benzyl aminopurine. No shoots regeneration was observed in both cultivars on MS media containing 0.2 mg $L^{-1}$ NAA + 0.0 mg $L^{-1}$ BAP. The growth regulators combination (2.0 mg $L^{-1}$ BAP+ 0.2 mg $L^{-1}$ NAA) was found most favorable in term of percentage calli regenerated and number of shoots per callus for SPTG-172 and the calli of cultivar K-399 showed best response to the growth regulators combination (MS + 1.0 mg $L^{-1}$ BAP + 0.2 mg $L^{-1}$ NAA) however, the percentage of calli regenerated were lower then SPTG-172. The roots developing ability of SPTG-172 and K-399 was similar on hormones free MS media.

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