Pak. J. Bot., 41(6): 2807-2816, 2009.

IN VITRO CLONAL MULTIPLICATION AND ACCLIMATIZATION OF DIFFERENT VARIETIES OF TURMERIC (*CURCUMA LONGA* L.)

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Abstract

A rapid propagation and acclimatization method of three different varieties of turmeric (Faisalabad, Kasur and Bannun) was optimized in this study**.** Sprouted rhizome buds of these varieties were collected and surface sterilized. The excised rhizome bud explants (1.0 cm) were cultured on MS medium supplemented with different concentrations and combinations of cytokinin and auxins. The frequency of shoot induction was 70, 60 and 75 in Faisalabad, Kasur and Bannun varieties respectively. The number of shoots per explant increased with increased BAP concentration while shoot length decreased. These regenerated shoots were further multiplied by sub culturing on fresh medium after 30 days. The regenerated plants of all varieties were transferred to different mixtures of compost for acclimatization. Best hardening response was obtained in Sand + Soil + Peat (1:1:1) after three week of transplantation in glass house. These hardened plants were subsequently shifted into field.

Introduction

Turmeric (*Curcuma longa* Linn.) of the family *Zingiberaceae* is a perennial rhizomatous herb (Panda *et al*., 2007; Purseglove, 1972) and has a wide spread occurrence in the tropics of Asia and extends to Africa and Australia (Govindarajan, 1980). Its rhizomes are oblong, ovate, pyriform and short branched (Eigner & Scholz, 1990).

Turmeric is valued all over the world as a spice, colouring agent and also for its medicinal properties (Roses, 1999; Nasirujjaman *et al*., 2005). It improves the complexion and is useful in diseases of blood, leucoderma, scabies, inflammations (Arora *et al*., 1971; Satoskar, *et al*., 1986), ozoena, bad taste in the mouth, biliousness, dyspepsia (Thamlikitkul *et al*., 1989), ulcer (Vandau *et al*., 1998; Kositchaiwat *et al*., 1993), elephantiasis, snake-bite, smallpox, boils and sprains (Kiritikar & Basu, 1996). It is also helpful in liver problem, urinary discharges and bruises (Kiritikar & Basu, 1996). It has been used internally as a stomachic, tonic and blood purifier and externally in the prevention and treatment of skin diseases. Curcumin and other curcuminoids found in turmeric inhibit growth of various bacteria. The rhizome juice is used as anthelmintic as well as in asthma and gonorrhea (Tiwari & Agrawal, 2003).

Turmeric currently in Pakistan and other developing countries is mainly utilized as spice and culinary additive in curries and other local dishes. But no significant work has been done on its value added extract i.e., curcumin and Oleoresins. Although we are the 2nd largest producer of turmeric in the world but per acre yield and quality is pathetically low. Slow multiplication rate, limited availability of high yielding genotypes, expensive field maintenance of planting material and high susceptibility of turmeric to rhizome rot diseases necessitates application of tissue culture techniques as a solution to these problems (Khader, 1994; Nayak & Naik, 2006). The high susceptibility of this crop to

soft rot (caused by *Pythium myriotylum*, and *P. gramnicolum*) and bacterial wilt (*Pseudomonas solanacearum*) is a major constraint in the production of turmeric. This crop has been badly neglected and no significant work has been reported for improvement of this crop. Therefore very intensive and focused research and development programme is needed to overcome the chronic issue of production, yield and quality.

In this study improvement of turmeric has been undertaken by applying plant tissue culture for rapid propagation of different varieties. Since plant tissue culture is reliable technology for mass propagation, for rapid clonal multiplication and conservation of important plant species, making the stock disease free and improving the genetic base of product (Philip & Gamborg, 2005) therefore it is the primary tool for improvement of turmeric in this study. An effort was made to establish a protocol for the *In vitro* propagation of turmeric from rhizome buds.

Materials and Methods

Explant germination: Rhizomes of all varieties were procured from Ayub Agriculture Research Station Faisalabad. For germination of rhizomes of different varieties of turmeric, different techniques for germination were opted. Some rhizomes were kept in autoclaved sand, soil and some were grown without sand or soil in dark. After germination, these germinated rhizomes were used for further study.

Surface sterilization: Explants (Germinated rhizomes) of different turmeric varieties i.e. Faisalabad, Kasur and Bannun were first treated for sterilization with household detergent (Ultra Surf) for 5 minutes. This was followed by washing with tap water to remove all the traces of detergent. Explants were then immersed in 70% ethanol for 30-40 seconds. The explants were further sterilized with 20-50% sodium hypochlorite for 15 minutes. Then these were washed three times with autoclaved distilled water to remove all the traces of sodium hypochlorite.

Micropropagation: Apical meristems excised from sterilized buds of different varieties have been cultured on nutritional media supplemented with different hormones. Effects of these hormones on root initiation and shoot formation of *In vitro* cultures has been tested and media providing the best results were selected for each variety of turmeric. Initially explants were cultured on MS basal medium supplemented with various concentrations of cytokinins and auxins alone and in different combinations. The basal medium used for all the experiments were Murashige & Skoog (1962) mineral formulation containing standard salts and vitamins, 30g/l sucrose, 5.0 ml/l PPM (plant preservative mixture) and 1.5g/l phytagel. The pH of each medium was adjusted to 5.5±0.1 before adding phytagel and medium was autoclaved at 15 Ib/inch^2 for 20 min at 121ºC. Cultures were incubated at 25±1ºC with a photoperiod of 16 h at 2000-3000 lux light intensity of cool white fluorescent light.

Multiplication and rooting of microshoots: To standardize the medium for induction of multiple shoots, different concentrations of cytokinins alone or in combinations were used in MS medium. Observations on frequency of shoot formation, number of shoots/ explants and shoot height in cm were recorded at 30 days after establishing the culture.

Acclimatization of micro plants: After rooting, regenerated plants were transferred to small pots containing different combinations of sand, soil and peat under greenhouse conditions for 1-3 month. The hardened regenerants were then transplanted to the trial field for further growth and multiplication.

Statistical analysis: A completely randomized design with 3 replicates was used for the experiment. The data for each parameter were subjected to analysis of variance (ANOVA) using the COSTAT V.63: statistical software (Cohort software, Berkely, California). The mean values were compared with the least significant difference test following Dancun's new multiple range test at 5% level.

Results and discussion

Establishment of aseptic culture: The establishment of contamination-free culture is a difficult and laborious process as the sterilization of underground rhizomes was very difficult due to presence of different types of soil born pathogens in these rhizomes. Therefore in this study, the different methods of sterilization were used to get the optimum level of contamination-free cultures. We had better results by incorporating 70% ethanol during surface sterilization. The supplementation of 5.0 mg/l PPM (plant preservative mixture) in the medium was also proved helpful in getting contamination free cultures. By using these precautions, more than 70% explants remained contamination-free. The difficulty in establishment of contamination-free *in vitro* cultures of *C. longa* was also reported by many groups (Balachandran *et al*., 1990; Dekker *et al*., 1991; Nadgauda *et al*., 1978; Salvi *et al*., 2002; Shirgurkar *et al*., 2001; Sunitibala *et al*., 2001; Yasuda *et al*., 1988).

Rapid plant multiplication: The rhizome buds from all the varieties were inoculated on MS media containing varying combinations of cytokinins and auxins for 4 weeks to get optimal medium for each variety. Percentage of response of the explants to all combination was shown in Table 1. It is evident that among the different media used, the medium containing 3.0 mg/l BAP showed the best shoot induction in Faisalabad and Kasur variety. Bannun showed the maximum response in BAP+NAA (2.0 mg/l+1.0 mg/l) (Fig. 1a,b,c). Among the three varieties, Bannun showed the highest response of shoot induction (75%) followed by Faisalabad (70%) and kasur (60%).

Although small quantity of cytokinin may be synthesized by apical rhizome buds grown *In vitro*; however, its exogenous supply also stimulates and promotes shoot proliferation in most of the plants (Ammirato, 1986). It is found that cytokinin not only determined the regeneration response but also affected the mode of regeneration. Addition of BAP alone in lower concentration in the medium supported poor regeneration response and produced single shoots (Short, 1986) but the higher concentration (Panda *et al*., 2007) and combination with NAA stimulated high frequency of regeneration particularly the formation of multiple shoots (Nasirujjaman *et al*., 2005; George, 1993).

Table 1. Effect of various growth hormones on frequency of shoot initiation of different varieties of *Curcuma longa*.

S. No.	Varieties	MS media + growth regulators		Percentage of shoot induction (%)
1.	Faisalabad	BAP	1.0 mg/l	60
2.		″	3.0 ″	70
3.		BAP + NAA	2 ″+ 1″	50
4.		″ + ″	10µM +1µM	45
5.		TDZ	18 ″	35
6.		TDZ + BAP	1 ″+ 5 ″	50
7.		TDZ + NAA	2.3 ″+ 0.53 ″	30
9.	Kasur	BAP	1.0 mg/l	30
10.		″	3.0 ″	60
11.		BAP + NAA	2 ″+ 1″	54
12.		″ + ″	10 µM +1µM	25
13.		TDZ	18 ″	50
13.		TDZ + BAP	1 ″+ 5 ″	40
14.		TDZ+NAA	2.3 ″+ 0.53″	30
16.	Banun	BAP	1.0 mg/l	55
17.		″	3.0 ″	74
18.		BAP + NAA	2 ″+ 1″	75
19.		″ + ″	10 µM +1µM	33
20.		TDZ	18 ″	25
21.		TDZ+ BAP	1 ″+ 5 ″	45
22.		TDZ+NAA	2.3 ″+ 0.53″	35

The response was evaluated after four weeks.

For multiplication of induced shoots MS medium was supplemented with BAP alone ranging from 1.0-6.0 mg/l or in combination with 1.0 mg/l NAA (Table 2). As it is evident from Fig. 1a, Faisalabad variety showed maximum no. of shoots (6.67) in medium supplemented with 4.0 mg/l and minimum (3.23) in medium with 1.0 mg/l BAP (Fig. 1.g). Kasur variety exhibited maximum no. (5.34) in same medium and minimum no. of shoots (3.69) in 2.0 mg/l BAP. (Fig. 2) The lowest response of no. of shoots (3.02) was noticed in BAP 2.0 mg/l while the highest response (6.77) was observed in medium supplemented with BAP 5.0 mg/l in Bannun variety (Text Fig. 3) The addition of NAA with all concentration of BAP did not show any significant effect on no. of shots in all varieties (Figs. 1, 2, 3). The higher dosage of BAP decreased the rate of multiplication.

Concentration of BAP also influenced the shoot length. As for as the length of induced shoots and roots is concerned, the lower dosage of BAP gave the maximum response while the higher concentration reduced the shoot and root length. Table 2 clearly demonstrates the results. In BAP 1.0 mg/l the maximum shoot length was 5.57 in Faisalabad, 6.99 cm in Kasur and 4.01cm in Bannun varieties (Fig. 1h) while the minimum length was observed in higher dosage of BAP i.e 6.0 mg/l. No addition of auxin is required for initiation of roots as the roots emerged simultaneously with shoots (Fig. 1f-g). The data of root length was also in conformity with the shoot length. The addition of NAA with all concentration of BAP showed the reduction in shoot and root length in all varieties. It is inferred that with increase in BAP concentration the number of shoots per explants increased but shoot length decreased.

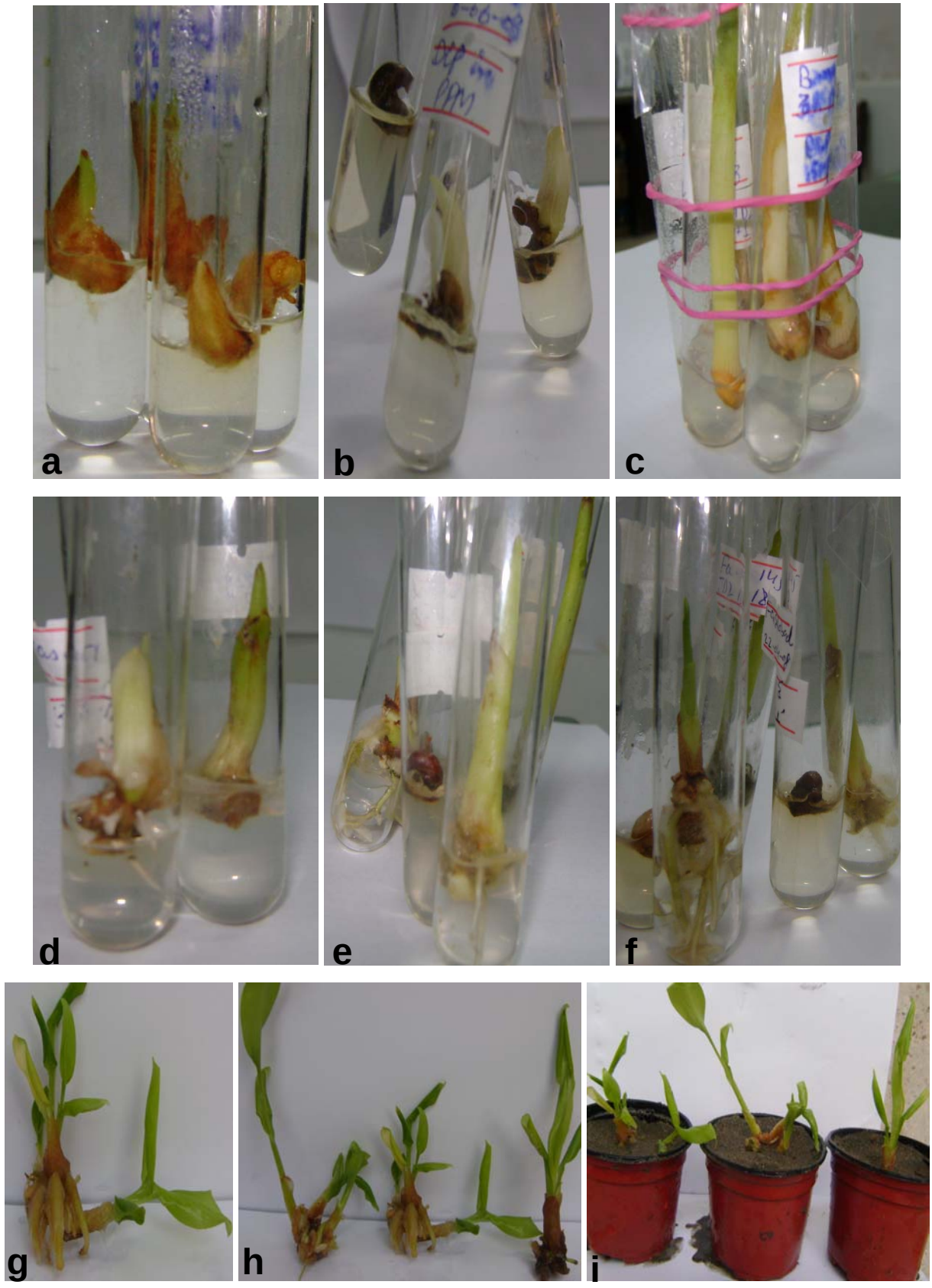

Fig. 1. Different stages of Micropropagation: **a)** Different explants of Kasur variety. **b)** Bannun variety. **c) & d)** Elongation of rhizomes e) Initiation of roots in MS +NAA 0.5mg/l in Kasur variety. **f)** Well developed roots in MS+2.0mg/l in Faisalabad variety. **g)** Full-fledged plantlet of Faisalabad variety **h)** Another view of full-fledged plantlet of Faisalabad, Kasur & Bannun variety. **i)** Acclimatization of mericlones of three varieties in sand.

Table 2. Effect of different concentrations of cytokinin and auxins on shoot and root length (cm) of different varieties of *Curcuma longa*.

S. No.	MS media + growth regulators (mg/l)	Faisalabad		Kasur		Bannun	
		Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)
1.	BAP 1.0	$5.57^{a} \pm 1.29$	$4.60^{a} \pm 0.05$	$6.99^{a} \pm 0.016$	$2.56^{d} \pm 0.12$	$3.94^{ab} \pm 0.1$	$2.51^{gh} \pm 0.063$
2.	" 2.0	$4.94^{b} \pm 0.005$	$3.19^{d} \pm 0.06$	$6.21^{b} \pm 0.021$	$2.58^{d} \pm 0.09$	$4.01^{a} \pm 0.12$	$2.64^{fg} \pm 0.19$
3.	" 3.0	$4.45^{c} \pm 0.004$	$3.41^{cd} \pm 0.07$	$5.90^{c} \pm 0.014$	$4.44^{ab} \pm 0.09$	$3.64^{b} \pm 0.06$	$2.37^{gh} \pm 0.07$
4.	" 4.0	$4.15^{d} \pm 0.01$	$3.67^{bc} \pm 0.071$	$4.23^{f} \pm 0.009$	$3.67^{c} \pm 0.07$	$2.41^{ef} \pm 0.05$	$3.59^{bc} \pm 0.052$
5.	" 5.0	$3.10^{f} \pm 0.04$	$3.34^{cd} \pm 0.10$	$3.22^{i} \pm 0.014$	$3.56^{c} \pm 0.1$	$2.24^{f} \pm 0.2$	$3.18^{de} \pm 0.14$
6.	" 6.0	$2.89^{g} \pm 0.005$	$3.25^{d} \pm 0.15$	$3.69^{h} \pm 0.004$	$4.17^{b} \pm 0.13$	$2.54^{def} \pm 0.12$	$3.84^{ab} \pm 0.07$
7.	BAP +NAA 1.0 "+ 1.0"	$5.48^{a} \pm 0.028$	$2.63^{e} \pm 0.10$	$4.52^{e} \pm 0.01$	$3.56^{c} \pm 0.077$	$3.24^{c} \pm 0.07$	$4.01^{a} \pm 0.10$
8.	" + " 2.0 "+ 1.0"	$5.45^{a} \pm 0.007$	$3.65^{bc} \pm 0.072$	$4.92^{d} \pm 0.005$	$3.43^{c} \pm 0.1$	$4.21^{a} \pm 0.06$	$3.61^{bc} \pm 0.07$
9.	" + " 3.0 "+ 1.0"	$4.64^{c} \pm 0.009$	$3.38^{cd} \pm 0.11$	$3.73^{h} \pm 0.006$	$3.56^{c} \pm 0.07$	$3.67^{b} \pm 0.045$	$3.41^{cd} \pm 0.16$
1.0	" + " 4.0 "+ 1.0"	$3.54^{e} \pm 0.065$	$4.71^{a} \pm 0.126$	$4.01^{g} \pm 0.122$	$4.74^{a} \pm 0.1$	$2.74^{de} \pm 0.11$	$2.95^{ef} \pm 0.115$
11.	" + " 5.0 "+ 1.0"	$2.25^{h} \pm 0.077$	$3.88^{b} \pm 0.09$	$2.91^{j} \pm 0.052$	$2.65^{d} \pm 0.1$	$2.61^{def} \pm 0.14$	$2.17^{h} \pm 0.091$
12.	" + " 6.0 "+ 1.0"	$2.17^{h} \pm 0.027$	$2.35^{e} \pm 0.012$	$2.61^{k} \pm 0.044$	$2.49^{d} \pm 0.02$	$2.85^{d} \pm 0.02$	$2.64^{fg} \pm 0.008$
	LSD	0.37	0.33	0.31	0.308	0.30	0.35

Each value is mean of three replicate with standard error (Mean± S.E) a,b,c: Mean with same superscript are not significantly different from each other at 5% level by Duncan's new multiple range test.

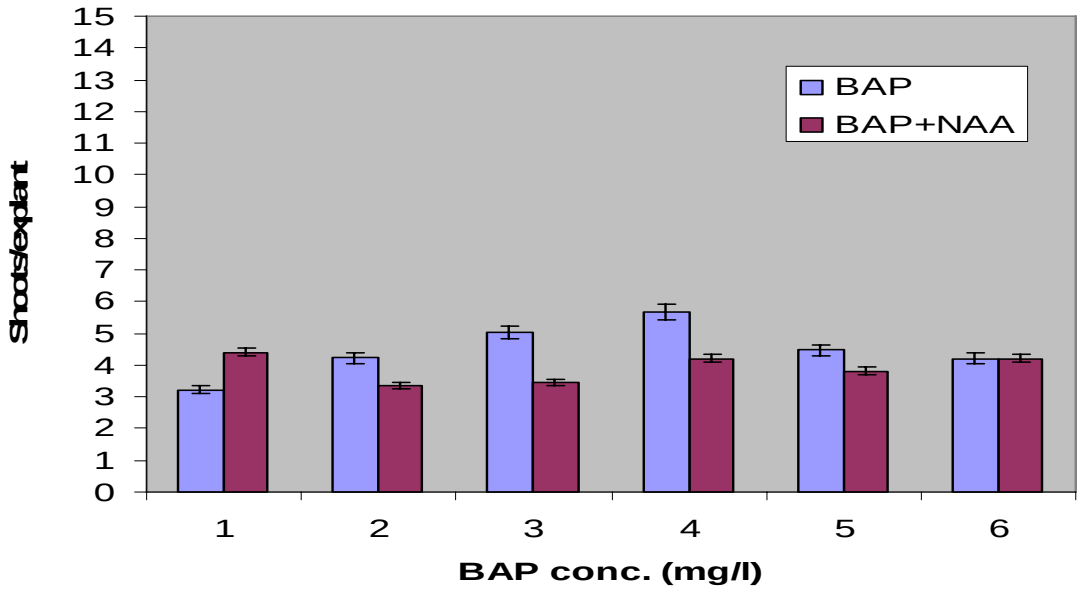

Fig. 1a. Effect of BAP alone and in combination with NAA on the no. of shoots of rhizome buds of Faisalabad variety.

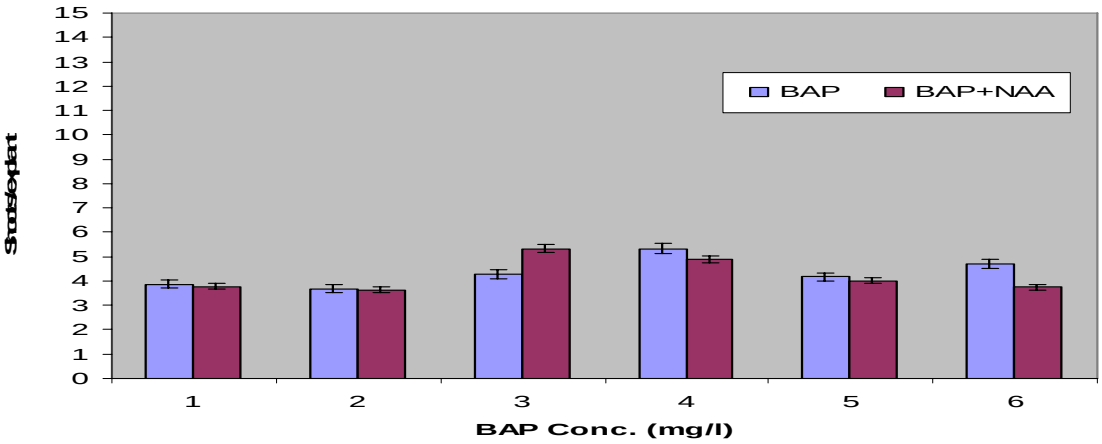

Fig. 2a. Effect of BAP alone and in combination with NAA on the no. of shoots of rhizome buds of Kasur variety.

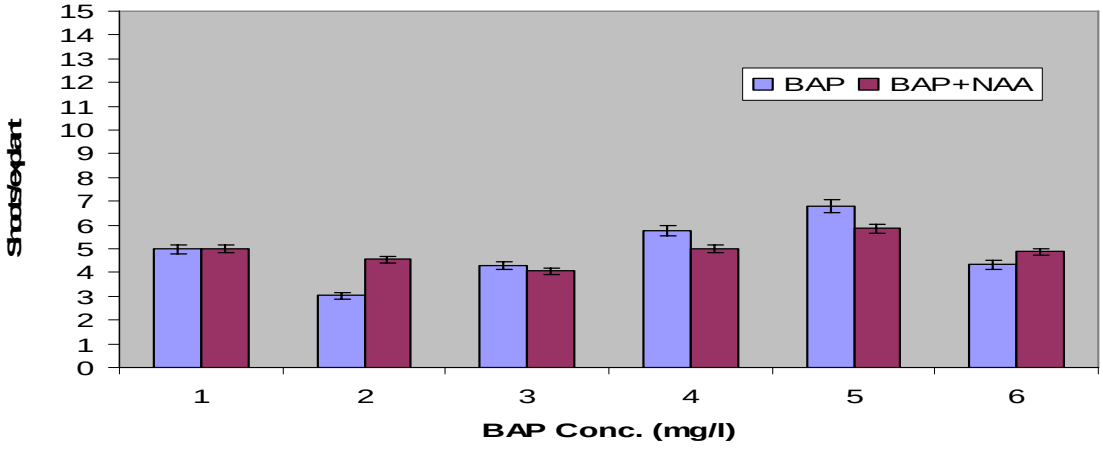

Fig. 3a. Effect of BAP alone and in combination with NAA on the no. of shoots of rhizome buds of Bannun variety.

The effect of BAP alone or combination with NAA was also well documented. Balachandran *et al*., (1990) reported a proliferation rate of 3.43 shoots/ explant after growing terminal buds on MS supplemented with 13.32 µM BAP for 4 weeks. Salvi *et al*., (2002) also reported shoot multiplication rates of 4.2, 3.5 and 6.6 shoots/buds following the culture of shoot-tip explants for 8 weeks in liquid medium supplemented with 1µM NAA and BA, Kin, or 2iP (10 µM each), respectively.

The ability of BAP to induce multiple shoots was also reported by different workers. Debergh and Zimmerman (1991) suggested that herbaceous plants are highly responsive to BAP treatments and most cultured herbaceous species produce robust, well-formed shoots suitable for further shoot proliferation. Chaudhary (1991) reported the effect of BAP on shoot proliferation of Indian rose and optimum shoot production in 2.5 mg/l BAP. Nasirujjaman *et al*., (2005) reported 6.7 multiple shoots of turmeric on MS medium supplemented with 4.0 mg/l BAP + 1.0 mg/l NAA within 8-10 days of inoculation. Panda *et al*., (2007) developed *in vitro* propagation protocol for *Curcuma longa* and gave 7.6 shoots by using medium with 3.0 mg/l BAP. While Prathanturarug *et al*., (2003) obtained 18.22 shoots/explants after 12 weeks by using 18.17 µM TDZ. Rahman *et al*., (2004) reported best shoot proliferation from rhizome bud explants in MS + 2.0 mg/l BAP and gave 6.2 average lengths of shoots per culture.

Acclimatization of plants under *ex vitro* conditions: In order to maximize the survival of *In vitro* derived plants, it is routine practice to acclimatize them under high levels of relative humidity (Short, 1991). *In vitro* conditions cause a variety of morphological abnormalities particularly of the stomata and cuticle, resulting in high mortality rate after transfer of plants to glass house or field conditions (Grout and Aston, 1977; Rahman *et. al*., 2004).

In present study *In vitro* grown turmeric plants were acclimatized in humid environment, following transfer to soil conditions. A simple method adopted in this study has facilitated the successful transfer of 70-80% of plants from *in vitro* to *ex vitro* conditions. After 30-50 days the potted plants were nurtured in glass house under complete sunlight for further acclimatization of plantlets. After another two weeks, these plants were transferred successfully into field. It was further observed that high humidity and moderate temperature greatly enhance the initial survival of potted and field grown plantlets. The different combination of compost also greatly affected the survival percentage of plantlets. Among the different combination tried, the best hardening response was observed in sand: soil: peat (1:1:1) combination. (Fig. 1i)

The results obtained in the present study could be highly significant. This efficient and reliable plant regeneration system via direct regeneration system can be exploited for improvement in yield and productivity through genetic transformation and other cellular techniques.

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(Received for publication 20 May 2009)