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In vitro Direct Regeneration of Three Indigenous Chickpea (*Cicer arietinum* L.) Varieties of Bangladesh

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Abstract

An *in vitro* direct plant regeneration protocol was developed for three locally grown chickpea (*Cicer arietinum* L.) varieties of Barichhola, namely BCh-2, BCh-4 and BCh-5. The best response towards multiple shoot regeneration was achieved from cotyledonary nodal explants on MSB_5 containing 4× MS micro-salts, 3.0 mg/l BAP and 0.04 mg/l NAA in all the three varieties of chickpea. In decapitated mature embryo axes explant best shoot regeneration was obtained on MS supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.2 mg/l NAA, 4 mg/l tyrosine, 880 mg/l $CaCl_2$ and 3800 mg/l KNO_3 in BCh-4 and BCh-5. Shoots regenerated on 1.0 mg/l Kn supplemented medium rooted efficiently on MS containing 0.2 mg/l BAP and 0.5 mg/ NAA in all the three varieties. Micrografting technique has been found to be a good alternative to *in vitro* rooting.

Introduction

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop globally (Van Rheenen 1991). It is considered as an important source of dietary proteins both for human and animals. Chickpea is valued for its nutritive seed (protein content: 22%) that provides the much needed protein to the underprivileged people in this subcontinent who cannot afford animal proteins. It is also found to improve soil fertility by fixing atmospheric nitrogen.

Chickpea now covers only 3% of the pulse cultivation area in Bangladesh occupying the fifth position among pulses (Bangladesh Bureau of Statistics 1999). This crop is characterized by low yield potential. Moreover, chickpea production in this country has declined substantially for the last several years because of some biotic and abiotic stresses. Among biotic stresses the most important fungal diseases are Botrytis gray mold disease (*Botrytis cinerea*), Alternaria blight (*Alternaria alternata*) and Fusarium wilt (*Fusariun oxysporum*). Some pests such as the pod borer, Bruchid beetles etc. are also severely affecting its yield. Therefore, there is an urgent necessity to develop disease and pest resistant cultivars of

chickpea and to increase productivity of chickpea in order to meet the growing need for this crop in Bangladesh.

Lower genetic variability, cross incompatibility and unavailability of resistant genotypes against biotic and abiotic stresses posed the major obstacles towards the improvement of this crop through conventional breeding. A number of attempts have been made in the past to improve chickpea varieties using tissue culture technique. But it has not yet been possible to develop resistant cultivars using tissue culture methods.

Under these circumstances, genetic transformation technique offers new hope, as specific genes from any source conferring desired traits can be introduced into a crop and can provide a solution to certain constraints that limit crop production and quality. This technique has been proved promising in the improvement of several other crops like maize, canola, cotton etc. and together with traditional breeding techniques it could also aid in improving chickpea yield and quality. *Agrobacterium*-mediated genetic transformation can be one of the methods of choice for chickpea as for years it has been used successfully for transformation of legumes and other dicotyledonous plants.

The most important pre-requisite of gene transfer technique is a robust, reproducible and efficient *in vitro* regeneration protocol. But, as legumes are generally recalcitrant *in vitro*, the success of chickpea regeneration and transformation are limited compared to other crops. There are a number of reports on chickpea regeneration *in vitro* (Riazuddin et al. 1988; Rao and Chopra 1989; Malik and Saxena 1992; Barna and Wakhlu 1994; Brandt and Hess 1994; Suhasini et al. 1994; Kumar et al. 1995; Murthy et al. 1996; Polisetty et al. 1997; Jayanand et al. 2003). However, the regeneration protocols described in various reports in the past tend to be irreproducible and unsatisfactory. Therefore, the *in vitro* regeneration protocol for chickpea needs to be improved further. Some regeneration experiments were carried out on Hyprosola and Binasola-2, the two varieties released by BINA (Bangladesh Institute of Nuclear Agriculture), Bangladesh (Sarker and Awal 1999). But no work has been done on indigenous Barichhola varieties, which are cultivated throughout this country.

It was with this background, attempts have been made to establish an efficient regeneration protocol suitable for Barichhola (BCh) so that the same, with or without modification may be applied to obtain chickpea transgenics of desired traits by *Agrobacterium*-mediated genetic transformation.

Materials and Methods

Seeds of three local varieties of chickpea (*Cicer arietinum* L.), namely Barichhola: BCh-2, BCh-4 and BCh-5 collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur were used in the present investigation.

Seeds were surface sterilized according to the protocol described by Sarker et al. (2003) and soaked overnight in sterile distilled water or cultured in water-agar medium for germination.

Two types of explants were used: (1) Decapitated mature embryo axes from overnight soaked seeds and (2) cotyledonary nodes taken from six - seven days old seedlings. The seeds were split open and mature embryo devoid of shoot and root meristems were taken as decapitated mature embryo axes explant; and decapitated mature embryo attached to the cotyledons was taken as cotyledonary node explant.

For regeneration of shoots, all explants were cultured on MS or MSB5/MS with B_5 vitamins (Gamborg et al. 1968) with various hormonal supplements, namely BAP, Kn, NAA, GA_3 and TDZ. In certain combinations, tyrosine was added in shoot regeneration medium and MS micro-salts and certain MS macro-salts, namely $CaCl_2$, KNO_3 and NH_4NO_3 were used in higher concentrations than the normal MS. For rooting, 3 - 5 cm long regenerated shoots were cultured on full or half strength MS solid or liquid medium supplemented with various concentrations and combinations of IBA and NAA. All the cultures were maintained under 16 hours photoperiod at 25 ± 2 ^{o}C.

Sometimes regenerated shoots were micrografted on to rootstocks taken from six days old aseptically germinated seedlings. The scion (shoot) base was given a "V" shaped cut and the upper part of the rootstock was split gently with a pointed scalpel. After placing the scion into the split of the stock, they were tied together with sterilized cotton thread. The grafted shoots were grown on half strength MS solid medium without any growth regulators. The plantlets with well-developed root system were transplanted in sterilized soil in small pots.

Results and Discussion

The mode of regeneration adopted in this present study was direct organogenesis. The regeneration response of cotyledonary node explants was better than embryo axes explant. No remarkable variation was observed among the varieties regarding shoot regeneration on a particular medium and hormonal combination.

The highest number of multiple shoots regenerated from decapitated mature embryo axes and cotyledonary node explants on MSB_5 containing 4× MS micro-salts, 3.0 mg/l BAP and 0.04 mg/l NAA (Tables 1 and 2). Kar et al. (1996) reported this combination as suitable for regeneration from decapitated mature embryo explant. In the present investigation, the above mentioned combination was proved to be the best for shoot regeneration from cotyledonary node explant (Figs. 2 and 3); but was not suitable for decapitated mature embryo explant.

Unlike the shoots regenerated from cotyledonary node explant, the mature embryo derived shoots were unhealthy, pale green, with a few leaves. Callusing was also occasionally observed on accidentally injured portions of the explant (Fig. 4). Moreover, vitrification of shoots was noticed under optimum cultural conditions and even on medium with increased agar content (9 - 10%) than normal solid medium.

Table 1. Effect of different media and hormonal combinations on regeneration of shoots from cotyledonary node.

Medium	Hormonal supplements (mg/l)				% of respon-sive explants	Mean No. of shoots/explant Varieties		
	BAP	Kn	NAA	GA_3		BCh-2	BCh-4	BCh-5
MS	0.5	-	-	-	93.3	8.0	8.5	8.2
MS	0.5	0.5	-	-	96.7	8.2	9.0	8.5
MS	0.5	0.5	0.2	-	96.7	9.0	9.5	9.0
MS	0.5	0.5	0.2	0.5	96.7	9.0	11.0	10.0
MS	-	1.0	-	-	96.7	3.2	3.5	3.3
MS	3.0	-	-	-	86.7	14	15.5	14.0
MSB_5 + 4× MS micro-salts	3.0	-	0.04	-	90.0	17.5	18.0	17.0

Healthy green shoots with numerous expanded leaves were obtained on MS containing 1.0 mg/l Kn both from embryo axes (Fig. 5) and cotyledonary node explants. But the number of shoots per explant was quite low, the maximum number being three shoots - regenerants per explant (Tables 1 and 2). This is in agreement with results of Fontanna et al. (1993), who used the same medium for shoot regeneration.

Previous reports on chickpea regeneration showed that TDZ was more effective in multiple shoot regeneration even in low concentrations (Murthy et al. 1996; Goerge and Eapen 1999; Senthil et al. 2004; Tewari-Singh et al. 2004). However, the results of the present investigation indicated that in none of the three varieties regenerated shoots developed with no elongated shoots even in the presence of 1.0 mg/l GA_3, although initially the number of regenerated shoots/decapitated mature embryo explants was as high as 8 - 9 shoots/ explant on 0.7 mg/l TDZ supplemented MS medium (Table 2).

Among all the media and hormonal combinations tested, the best response toward multiple shoot regeneration from decapitated mature embryo explant was observed in MS with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.2 mg/l NAA (Table 2) in

all the three varieties. The number of shoots was 5 - 7/explant, but regenerated shoots were weak and shoot tip decayed eventually.

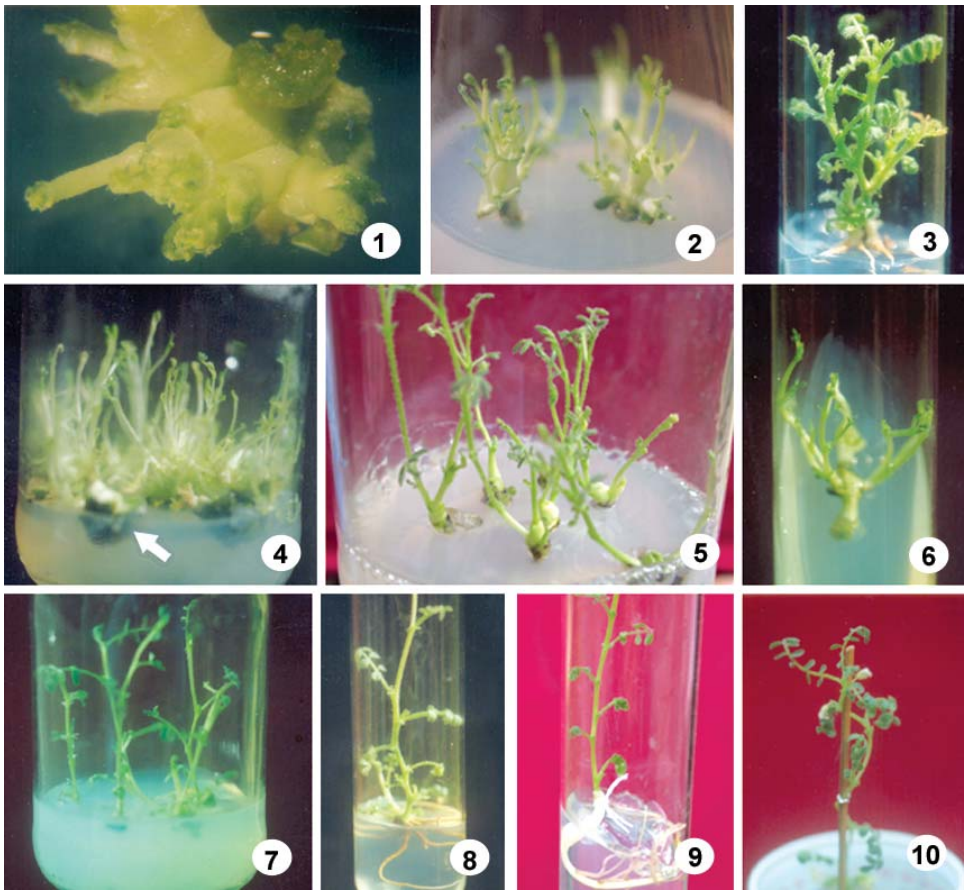
In wheat, jute and lentil, tyrosine was reported to have a positive effect in the development of healthy and elongated multiple shoots (Hossain 1993; Sarker and Biswas 2002; Sarker et al. 2003). Among all the concentrations of tyrosine added in MS medium, tyrosine at a concentration of 4 mg/l was most effective for the development of increased number of shoots (Table 2). It also promoted better responsiveness of explants (98.33%).

Table 2. Effect of different media and hormonal combinations on regeneration of shoots from decapitated mature embryo axes.

Medium	Hormonal supplements (mg/l)					% of respon-sive explants	Mean No. of shoots/ explant		
	BAP	Kn	NAA	GA_3	TDZ		Varieties		
							BCh-2	BCh-4	BCh-5
MS	0.5	-	-	-	-	95.0	5.0	6.0	5.5
MS	0.5	0.5	-	-	-	96.7	5.8	6.5	5.8
MS	0.5	0.5	0.2	-	-	96.7	5.8	7.0	6.0
MS	-	-	-	1.0	0.7	95.0	7.8	9.0	8.0
MS	-	1.0	-	-	-	96.7	2.5	3.0	3.0
MS	3.0	-	-	-	-	86.7	11.0	11.5	11.0
MSB_5 + 4× MS micro-salts	3.0	-	0.04	-	-	88.8	13.0	14.0	13.5
MS + 4 mg/l tyrosine, 2× $CaCl_2$, 2× KNO_3	0.5	0.5	0.2	-	-	98.3	6.0	8.0	7.0

Ye et al. (2002) reported that the double concentration (2×) of $CaCl_2$ than normal MS medium could suppress decay of shoot tip in lentil. In the present study, 2× $CaCl_2$ (880 mg/l) and some other macro-salts namely, 2× KNO_3 (3800 mg/l) and 2× NH_4NO_3 (3300 mg/l) was used in MS medium containing 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA and 4.0 mg/l tyrosine to see their effect on shoot health and in the reduction of shoot- tip decay. Among all the combina-tions, 2× $CaCl_2$ and 2× KNO_3 proved to be the best for regeneration and development of healthy and leafy green shoots from decapitated mature embryo explant (Figs. 6 and 7) and in overcoming shoot tip necrosis in BCh-4 and BCh-5. This treatment reduced shoot tip decay in BCh-2, but did not stop it totally.

Thus, the most suitable medium for direct shoot regeneration from decapitated mature embryo axes was MS medium containing 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA, 4.0 mg/l tyrosine, 2× $CaCl_2$ (880 mg/l) and 2× KNO_3 (3800 mg/l) (Fig. 11.).



Figs. 1-7: 1. Stereomicroscopic view of shoot initiation from decapitated mature embryo. 2-3. Multiple shoots regenerated from cotyledonary node. 4. From embryo axes on MSB_5 + 4× micronutrients + 3 mg/l BAP + 0.04 mg/l NAA in BCh-4 (callus shown by arrow). 5. Regenerated shoots on MS + 1.0 mg/l Kn in BCh-4. 6. Multiple shoots. 7. Elongated shoots regenerated from decapitated mature embryo axes on MS + 0.5 mg/l BAP + 0.5 mg/l Kn + 0.2 mg/l NAA + 880 mg/l $CaCl_2$ + 3800 mg/l KNO_3 in BCh-5. 8. Development of roots on MS + 0.2 mg/l IBA+ 0.5 NAA at the base of shoots regenerated on MS + 1 mg/l Kn supplemented medium in BCh-5. 9. Micrografted shoots of BCh-4. 10. A plant with flower (arrow) in pot.

There are a few publications reporting successful rooting of chickpea (Singh et al. 1982, Davis and Foster 1982). In recent reports, isolated shoots were successfully rooted in rooting media containing different concentrations of auxin, provided these shoots were previously regenerated in media containing either TDZ or Kn (Fontanna et al. 1993; Jayanand et al. 2003; Fratini and Ruitz 2003). Shoots that regenerated in BAP containing

media showed poor rooting response as reported by a number of authors (Kar et al. 1996; Polowick et al. 2004) and in most of the cases, rooting was accomplished through very difficult media pathways (Polowick et al. 2004). All of these reports indicate that, rooting of chickpea is extremely difficult. Same kind of difficulty was also observed in this present investigation.

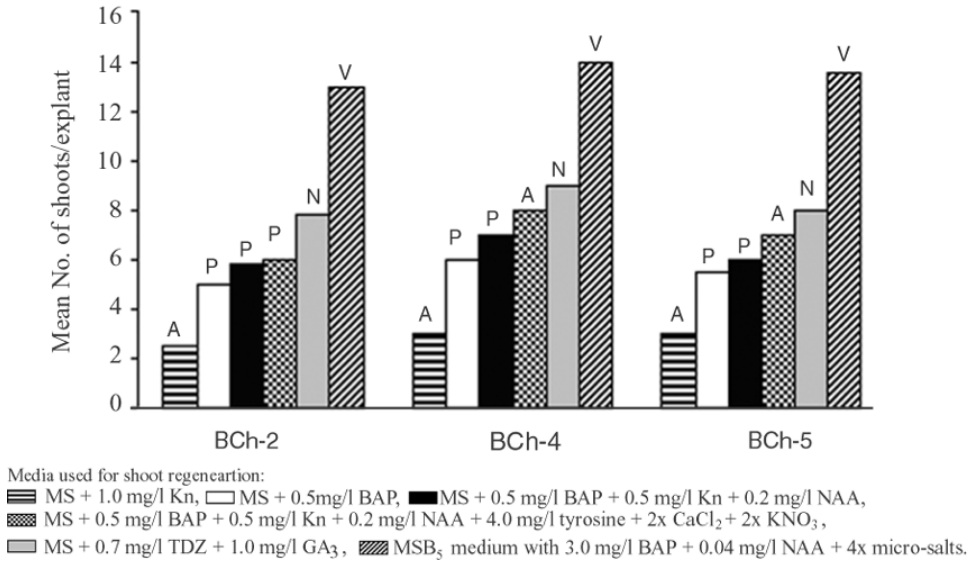


Fig. 11. Effect of different media and hormonal combinations on regeneration of shoots from decapitated mature embryo explants and shoot health in different varieties (Shoot health status: **A** = Shoot tip decay absent, **P** = Shoot tip decay present, **V** = Unhealthy vitrified shoots and **N** = No shoot elongation.

Only those shoots, which were developed in 1.0 mg/l Kn supplemented media rooted successfully in 0.2 mg/l IBA and 0.5 mg/l NAA supplemented MS (Fig. 8) and these shoots survived well upon transplantation (Fig. 10). In this rooting medium, only a few shoots that regenerated in 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA with or without tyrosine developed roots but this rooting response was not satisfactory at all.

Shoots that failed to develop roots were subjected to micrografting on rootstocks of *in vitro* germinated seedlings (Fig. 9). Most of the recent work on chickpea employed this technique for the production of plantlets when normal rooting was unsuccessful (Krishnamurthy et al. 2000; Sarmah et al. 2004; Senthil et al. 2004). In the present study, the percentage of shoots survived upon grafting was 30 - 41.67. This experiment demonstrated that micrografting could be applied as an alternative to obtain healthy root system for the development of plants *in vitro*.

The *in vitro* shoot regeneration procedure described in the present study is rapid, efficient and reproducible and can be used for locally grown chickpea varieties. As rooting in chickpea is extremely difficult, further refinement of the rooting technique is necessary. Also as an alternative method, the technique of *in vitro* grafting (Picardt et al. 1991; Gulati et al. 2001) can be applied to develop an efficient rooting system for this crop. In future, the regeneration protocol described here can be used for production of transgenic chickpea plants with desired traits.

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