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***In vitro* Plant Regeneration of Four Local Varieties of Chickpea (*Cicer arietinum* L.) Grown in Bangladesh**

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Abstract

In vitro regeneration system was developed through direct organogenesis from decapitated mature embryo explants of locally grown four chickpea varieties, namely, Barichhola-4, Hyprochhola, Binachhola-3 and Binachhola-4. Best response towards multiple shoot regeneration was obtained on MS medium supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.2 mg/l NAA along with double concentrations of CaCl₂ and NH₄NO₃. However good shoot health and expanded leaf was found on MS medium containing 1.0 mg/l kn. Apart from this, few experiments were conducted with decapitated embryo attached cotyledon. Using this explants highest number of multiple shoots were obtained on MSB medium containing 4× micronutrients of MS medium with 3.0 mg/l BAP and 0.04 mg/l NAA in all four varieties. Shoots regenerated on 1.0 mg/l kn supplemented medium showed good response towards rooting on MS medium supplemented with 0.2 mg/l IBA in all four varieties. It was observed that micrografting is an alternative technique to *in vitro* rooting in chickpea.

Key words: *In vitro* regeneration, Decapitated embryo, Chickpea.

Introduction

Chickpea is considered as an important pulse crop in Indo-Pak-Bangladesh subcontinent and consumed by majority of the people of these countries. Chickpea is rich in protein (generally high in lysine) and especially important for those countries where malnutrition is associated with acute shortage of animal protein production (Bressani 1973). Along with the proteins it also supply a good proportion of carbohydrates, fats and minerals. It improves soil fertility by fixing atmospheric nitrogen.

Chickpea is highly nutritious and required minimal inputs for its cultivation. However, this crop is characterized by low yield potential. The national productivity and per capita availability of this crop is very low in Bangladesh (Khan *et al.* 1981) 7g/day, which is compared to 49 g/day in India (Johl 1984). A number of biotic and abiotic stresses are severely affecting full realization of the yield potential of this crop. Among the biotic stresses the most important fungal diseases are Botrytis gray mold (*Botrytis cinerea*), Fusarium wilt (*Fusarium oxysporum*), Alternaria blight (*Alternaria alternata*), fungal blight (*Ascochyta rubici*), dry root rot (*Rhizoctonia balticola*), and collar rot (*Sclerotium rolfsii*). Botrytis gray mold, the most damaging foliar disease of

chick pea in Bangladesh, caused a substantial decline in chick pea production over the past decade. Some pests such as *Heliothis sp.*, *Callosobruchus chinensis* and *C. maculatus* causes much damage of seeds in storage condition.

Therefore, to obtain desired performance like disease and pest resistance cultivars, improvement of this crop is essential. In the past several attempt was made to develop disease resistant high yielding varieties of chickpea. However, due to cross-incompatibility and hybrid sterility it has not been possible to develop such improved chickpea varieties through conventional breeding methods.

Under this circumstances, genetic transformation technique offers new hope, as specific genes from any source conferring desired traits can be introduced into a crop to overcome certain constraints that limit crop production and quality (Gardner 1993). *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. Efficient *in vitro* plant regeneration system is required for successful crop improvement programs through genetic transformation.

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Several attempts have been made to establish *in vitro* regeneration protocol on chickpea. (Riazuddin *et al.* 1988, Rao and Chopra 1989, Malik and Saxena 1992, Barna and Wakhlu 1993, 1994, Suhasini 1994, Dineshkumar *et al.* 1995, Kumar *et al.* 1995, Murthy *et al.* 1996, Polisetty *et al.* 1997, Jayanand *et al.* 2003).

However, the *in vitro* regeneration protocols developed by the researchers in the past tend to be irreproducible (Sarker *et al.* 2005). Therefore, the *in vitro* regeneration protocol for chickpea needs to be improved further. There are some reports on some Bari Chhola varieties released by BARI (Bangladesh Agricultural Research Institute), Bangladesh (Sarker *et al.* 2005); some Bina chhola varieties released by BINA (Bangladesh Institute of Nuclear Agriculture), Bangladesh (Sarker and Awal 1999). But no work has been done combinedly with Bari chhola and Bina chhola which are cultivated throughout this country. With this background, attempts were made to establish an efficient regeneration protocol in different chickpea varieties of Bangladesh.

Materials and Methods

Four local varieties of chickpea (*Cicer arietinum* L.) were used in the present investigation namely Barichhola- 4, Hyprochhola, Binachhola-3, Binachhola-4. Among the seeds of these four varieties of chickpea (*Cicer arietinum* L.) Barichhola- 4 was collected from BARI, Joydebpur, Gazipur and other three varieties namely Hyprochhola, Binachhola-3 and Binachhola-4 were collected from BINA, Mymensingh.

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

Decapitated mature embryo axes and decapitated mature embryo attached to the cotyledons were used as explants from overnight soaked seeds. The seeds were split open and mature embryo devoid of shoot and root meristems were taken as decapitated mature embryo axes explants.

For shoot initiation and development all the explants were cultured on MS (Murashige and Skoog, 1962) or MSB (MS salts and B₅ (Gamborg *et al.* 1968) vitamins) medium with various hormonal supplements namely BAP, Kn, NAA, In certain combinations, MS macrosalt namely CaCl₂ and NH₄HO₃ were used in higher concentrations than those in

normal MS. For rooting, 3-5 cm long regenerated shoots were cultured on full or half strength of MS medium supplemented with various concentrations of IBA. All the cultures were maintained under 16 hours photoperiod at 25 ± 2°C.

Sometimes healthy shoots, which were unable to induce roots, were grafted onto rootstocks taken from aseptically germinated 6 days old seedlings. The upper part of the rootstock was made a split cut with the help of a fine pointed scalpel and the cut of lower part of the scion (shoot) was made "V" shaped. After placing the scion onto the stock, thin cotton threads were used to tie the attachment region. The entire operation was done inside a laminar flow cabinet. Grafted shoots were cultured in test tubes containing ½ MS medium without any growth regulators. The plantlets with well-developed root system were transplanted in sterilized soil in small pots.

Results and Discussion

For *in vitro* regeneration of shoots two type of explants like decapitated embryo axes (DE) and decapitated embryo attached with cotyledon (DEC) were used. No remarkable variation was observed among the varieties regarding shoot regeneration on a particular medium and hormonal combination.

In the present study, different concentrations of BAP, Kn, NAA, GA₃ were used singly or in combinations in MS and MSB medium to observe their effect on initiation and development of shoots. In all the four varieties maximum number of multiple shoots regenerated from both DE and DEC explants on MSB medium containing 4× microsalts, 3.0 mg/l BAP and 0.04 mg/l NAA. Kar *et al.* (1996) first reported this combination in case of mature embryo axes explant. In the present experiment, it was observed that the above combination was the best for DEC explant (Fig. 2,3 and 4), but not suitable for DE explants, as shoot regenerated from this explants were thin, pale green with very few leaves and sometimes caused callus formation.

Healthy, green shoots with expanded leaves were obtained on MS medium containing 1.0 mg/l Kn in case of both DE and DEC explants in all varieties of chickpea (Figs. 5, 6 and 7). But number of shoots per explant was low, maximum three shoots per explant were observed (Table I). Fontanna *et al.* (1993) first used this medium composition.

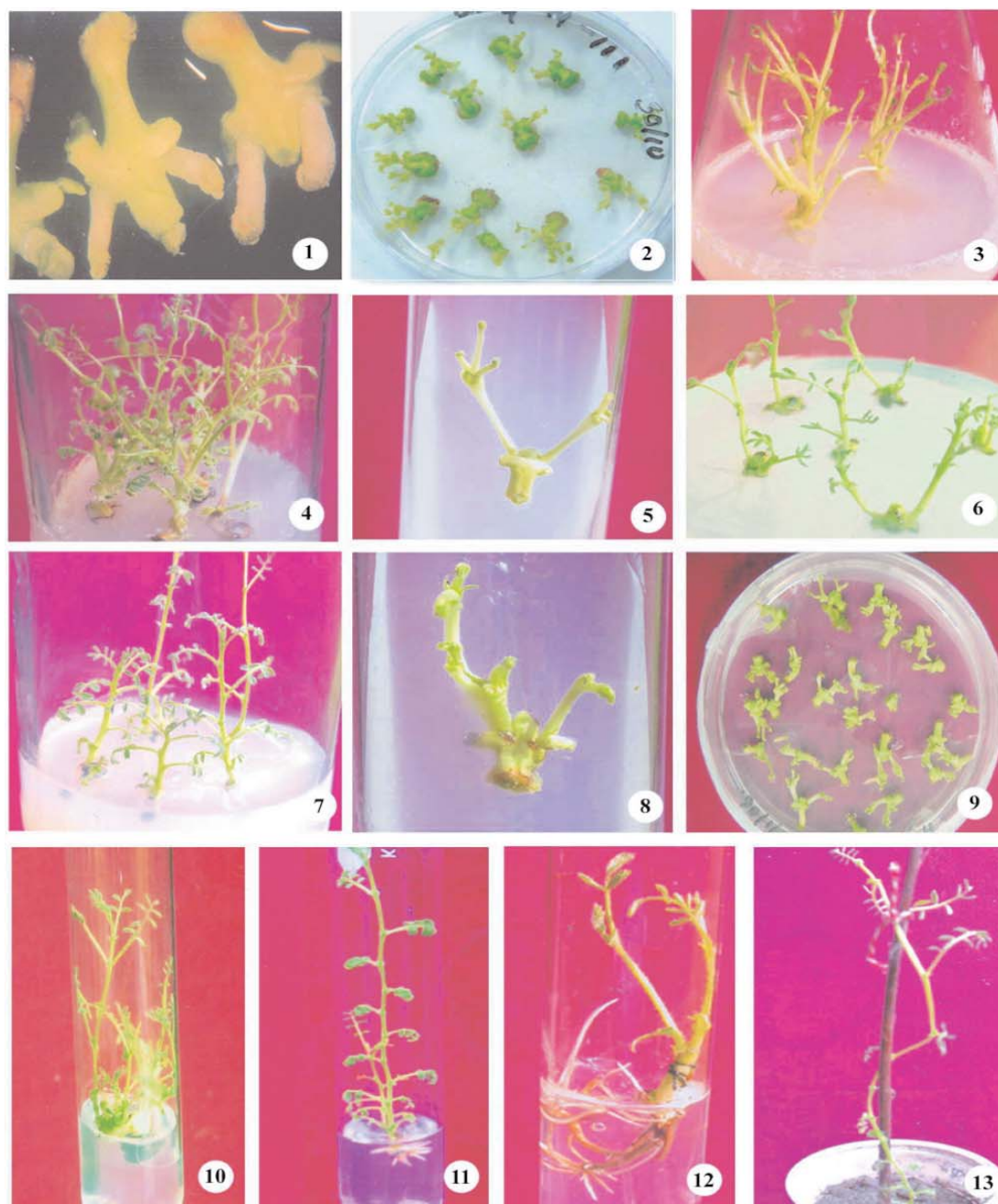


Fig. 1-13. Plant regeneration and acclimatization of chickpea. 1. Stereomicroscopic view of shoot initiation from decapitated embryo (DE). 2. Multiple shoot regeneration from DE of Binachhola-4 on MSB medium with 4x micronutrients, 3.0 mg/l BAP and 0.04 mg/l NAA. 3-4. Multiple shoot formation of Barichhola-4 from decapitated embryo attached cotyledon (DEC) in same medium. 5. Shoot initiation from DE of Binachhola-4 on MS medium with 1.0 mg/l Kn. 6-7. Shoot elongation of Barichhola-4 and hyprochhola in the same medium. 8-9. Multiple shoot initiation from DE of Barichhola-4 and Binachhola-4 on MS medium with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.2 mg/l NAA with 2x CaCl_2 and 2x NH_4NO_3 . 10. Shoot elongation of Hyprochhola in same medium. 11. Root initiation on MS medium with 0.2 mg/l IBA in Hyprochhola. 12. *In vitro* grafting in Barichhola-4. 13. Acclimatization of Hyprochhola in pot soil.

Krishnamurthy *et al.* (2000) used MS medium containing 0.5 mg/l BAP for shoot regeneration using embryo axes explants. Similar concentration was used in this experiment and observed that regenerated shoots were initially pale green with very few developing leaves. In a separate set of experiments, 0.5 mg/l BAP, 0.5 mg/l Kn alone and in combination with NAA was applied in MS medium for shoot regeneration and development. Sarmah *et al.* (2004) used similar type of hormonal combinations in shoot regeneration medium and reported successful regeneration. Among all media and hormonal combinations tested the best response was observed in MS medium supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA from decapitated mature embryo explants in all four varieties. The mean number of shoots was 6-7.5 per explant, but shoot tip death was observed eventually.

Ye *et al.* (2002) reported that MS medium with double concentration ($2\times$ normal MS medium) of CaCl_2 could overcome shoot tip death in lentil. In the present investigation, two fold concentration ($2\times$ normal medium) of CaCl_2 (880 mg/l) and some other macro-nutrients, e.g. potassium nitrate (3800 mg/l) and ammonium nitrate (3300 mg/l) were used singly or in combination in shoot regeneration medium. Among the various combinations of macro-salts, $2\times\text{CaCl}_2$ (880 mg/l) and $2\times\text{NH}_4\text{HO}_3$ (3300 mg/l) was proved to be the best for regeneration and development of healthy leafy green shoots and in overcoming shoot tip necrosis. Only Binachhola-3 failed to reduce shoot tip death. Other three varieties showed comparatively better response on this medium. Thus the most suitable media for direct shoot regeneration from DE explant was MS medium supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.2mg/l NAA, $2\times\text{CaCl}_2$

(880 mg/l) and $2\times\text{NHNO}_3$ (3300 mg/l) (figs. 8,9 and 10) in all four varieties(Table I).

There are very few publications reporting successful rooting of chickpea (Singh *et al.* 1982, Davis and Foster 1982). It has been reported that isolated shoots were successfully rooted if those shoots were regenerated in MS medium containing Kn (Fontanna *et al.* 1993, Jayanand *et al.* 2003, Fratini *et al.* 2003). Shoots regenerated on BAP containing medium showed lower number of root formation (Kar *et al.* 1996, Polowick *et al.* 2004) and in most 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. Similar situation was also observed in the present investigation. Those shoots that were developed in 1.0 mg/l Kn supplemented media rooted successfully (Fig. 11) than other media combinations in 0.2 mg/l IBA supplemented with full strength of MS medium. Hyprochhola showed the best rooting response (Table II). The plantlets with well developed roots were transferred to small plastic pots. After proper hardening they were established in field condition (Fig. 13).

The seasonal effect of the four varieties was observed. Shoots regenerated on MS medium supplemented with 0.2 mg/l IBA was cultured once in every month. It has been observed that maximum root induction was found when the shoots were cultured during the month of December to February. It was also observed that there was no root induction during the month of June-August. The results showed that there is a significant effect of seasons on the root induction of chickpea. Those shoots that failed to develop roots were subjected to micrografting on root stocks of *in vitro* germinated seedlings following the report of Krishnamurthy

Table I: Best hormonal combinations for shoot regeneration from decapitated mature embryo axes explants

Media + Hormonal supplement (mg/l)	Varieties	Days required for shoot initiation	Explants showed regeneration response (%)	Mean no. of shoots/explant
MS + 1 mg/l Kn	Barichhola- 4	6-8	95.00	2.5
	Hyprochhola	5-7	96.66	3.0
	Binachhola- 3	6-8	96.66	2.0
	Binachhola- 4	6-10	95.00	2.5
MS + 0.5 mg/l BAP+ 0.5 mg/l Kn+ 0.2 mg/l NAA+	Barichhola- 4	7-10	96.66	6.5
	Hyprochhola	7-10	96.66	7.0
	Binachhola- 3	8-10	98.33	6.0
	Binachhola- 4	8-10	95.00	7.5
2X CaCl_2 + $2\text{XNH}_4\text{NO}_3$				

Table II: Effect of 0.2 mg/l IBA on semi-solid MS medium for root induction from regenerated shoots of all four varieties

Shoot regenerated media	Varieties	No. of shoots inoculated for root induction	No. of responsive shoots	Days to root induction	No. of roots/explant
MS medium with Kn	Barichhola-4	30	19	7 - 16	3 - 5
	Hyprochhola	30	19	7 - 15	4 - 8
	Binachhola-3	30	15	7 - 16	3 - 6
	Binachhola-4	30	10	13 - 20	2 - 5
MS medium with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.2 mg/l NAA, 2x macronutrients	Barichhola-4	20	4	14 - 20	1 - 2
	Hyprochhola	20	6	12 - 20	2 - 3
	Binachhola-3	20	4	14 - 22	2 - 3
	Binachhola-4	20	2	18 - 25	2 - 3

et al. (2000), Sarmah *et al.* (2004) and Senthil *et al.* (2004). In the present study, among the four varieties Barichhola-4 showed the maximum rate (46.66%) of successful grafting.

Conclusion

The results of the present investigation demonstrated the establishment of a reliable *in vitro* regeneration protocol from decapitated mature embryo axes and decapitated embryo attached with cotyledon explants for four selected Bangladeshi chickpea varieties. This protocol may be effectively used for the improvement of different chickpea varieties through *Agrobacterium*-mediated or any other suitable methods of genetic transformation.

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