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In vitro clonal propagation of a male sterile line in Chilli crop

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Abstract A high frequency simple method of plant regeneration has been developed in male sterile line MS 12 of chilli. Different factors affecting plant regeneration were studied i.e., age of the explant, wounding of the cotyledons and the media composition. Cotyledons of 8-day old *in vitro* grown seedlings were excised and cultured on MS media supplemented with different concentrations and combinations of BAP, kinetin and IAA. The frequency of plant regeneration, days taken to plant regeneration and number of shoots per explant were influenced by age of explants, wounding and concentrations of growth regulators added to the media. Twenty four day old wounded cotyledons recoded maximum plant regeneration (77.77 %), minimum days to plant regeneration and maximum number of shoots (20.00) on MS media supplemented with BAP (9mg l^{-1}), kin (2 mg l^{-1}) and IAA (2 mg l^{-1}) which declined significantly with either decrease or increase from this optimum level. The shoots when attained approximately 2 cm height were sub-cultured onto the ½ strength MS medium supplemented with IBA (2mg/l) for one week and subsequently transferred to the ½ MS hormone free medium on which simultaneous shoot elongation as well as root formation took place. The plantlets thus obtained were transferred to poly bags containing FYM:sand:soil in the ratio of 1:1:1 under glass house conditions for establishment..

Keywords: Growth hormones, *Capsicum annuum* (L.), organogenesis, tissue culture

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Introduction

Chilli (*Capsicum annuum* L.) is an indispensable ingredient employed for food preparation in the world and an important product utilized in the pharmaceutical, food, cosmetic and poultry industries. India is the world's largest producer of chillies producing over 25 percent of the world's production, closely followed by China (24.00 percent), Spain (17.00 percent) and Mexico (8.00 percent). Due to the strong domestic demand in India only about 4 percent of total production is exported (Juan, 2009). To meet the domestic as well as foreign market demand of chilli development of superior genotypes is among few options available.

Chilli is a cross pollinated plant with a high level of heterogeneity in seed population (Tanksley, 1984). Such variability is undesirable in commercial chilli seed production because of a high proportion of offtypes. Superiority of F_1 hybrids have been reported for fruit yield, earliness, high productivity, high fruit weight and dry recovery (Hundal, 1995). Chilli cultivation in Punjab was revolutionized by evolution of chilli hybrids CH_1 and CH_3 at Punjab Agricultural University, Ludhiana, which involves genetic male sterile line MS 12 as female parent. The major bottleneck in this line is the occurrence of 1:1 ratio of fertile: sterile plants which require rouging of fertile plants at the time of anthesis by observing each and every individual (Hundal and Khurana, 1993). If the asexual reproduction of elite stocks can be done using biotechnological tools, the tedious rouging process can be avoided and seed yield per unit area can be increased.

Moreover, red pepper does not have natural ability for vegetative propagation. Therefore, *in vitro* propagation methods may be used for its clonal multiplication. Several tissue culture protocols have been described (Phillips and Hubstenberger, 1985; Agrawal *et al.*,. 1989; Ochoa-Alejo and Ireta-Moreno, 1990; Arroyo and Revilla, 1991; Valera-Montero and Ochoa-Alejo 1992; Ebida and Hu 1993; Ezura *et al.*, 1993; Christopher and Rajam, 1994; Binzel *et al.*, 1996; Ahmad *et al.*, 2006). Additionally, achieving the elongation of *in vitro* formed shoot buds in itself poses a considerable challenge because the ill-defined buds or shoot like structures either resist elongation or produce rosettes of distorted leaves which generally do not produce normal shoots (Arroyo and Revilla, 1991; Valera-Montero and Ochoa-Alejo, 1992). This obstacle is related to the high diversity of the existing genotypes, which results in a great variety of responses to *in vitro* tissue culture. Earlier, meager work has been done on clonal propagation of male sterile lines which would facilitate the 100 percent genetically pure plant stand as well as utilization of male sterile plants in other hybridization programmes. Therefore, in the present investigation following factors were studied i.e. age of seedlings, wounding of cotyledons and the media composition.

Materials and Methods

The seeds of chilli male sterile line MS 12 were surface sterilized with 75 percent commercial bleach for 20 minutes followed by four to five rinses in sterile water and germinated on half strength (inorganic salts strength reduced to half) (Murashige and Skoog, 1962) media containing thiamine HCl (0.1 mg l^{-1}), pyridoxine HCl (0.5 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), myo-inositol (100 mg l^{-1}), sucrose (3.0 g l^{-1}) and agar (0.8 %). The pH of media was adjusted to 5.8 by adding 1N NaOH/ 1N HCl solution drop-wise. The *in vitro* germinated seedlings served as an explant source for plant regeneration studies. Cotyledons were excised from 12, 18, 24 and 30 day old *in vitro* grown seedlings. Cotyledons were wounded under laminar air flow cabinet with the help of sterilized needles, scalpels and forceps. These explants were inoculated onto MS media supplemented with combinations and concentrations of BAP, kinetin and IAA (Table 1). The media was first dispensed into glass jars (100 ml/jar) when still hot. These glass jars were properly capped before autoclaving at 1.05 kg cm^{-1} square pressure and 121°C temperature for 22 minutes. To prevent the inoculation of microbes, all decontamination procedures and aseptic manipulations were performed in a laminar flow hood (Kreider, 1968). The experiment was repeated five times. The number of explants per genotypes varied from 100-200. The observations were recorded on percent plant regeneration, days taken to shoot regeneration and number of shoots per culture. The data were analyzed following the computer software package CPCS-I using factorial CRD design (Singh and Cheema, 1985).

Results and Discussion

Percent Plant Regeneration

Although, regeneration in chilli was successfully performed for variety of cultivars for many years (Phillips and Hubstenberger, 1985; Agrawal *et al.*,. 1989; Ochoa-Alejo and Ireta-Moreno, 1990; Arroyo and Revilla, 1991; Valera-Montero and Ochoa-Alejo 1992; Ebida and Hu 1993; Ahmad *et al.*, 2006). but a meager work has been done on micropropagation of chilli male sterile line. Therefore, miropropagation of chilli male sterile line was undertaken to determine the *in vitro* morphogenic response of cultured explants as affected by different factors. *In vitro* plant regeneration is the result of interplay of factors including source of explant, culture medium and plant growth regulators concentration and culture conditions (Bhatia *et al.*, 2004). Percent plant regeneration varied significantly with the age of the explants, wounding of explants as well as media composition. The data presented in Fig. 1 and Fig. 2 on wounded and unwounded cotyledons, respectively, indicated that lower level of growth regulators did not enhance per cent plant regeneration in all the age groups which reached to a maximum in twenty four day old cotyledons on MS 5 media supplemented with BAP (9mg l^{-1}), kin (2 mg l^{-1}) and IAA (2 mg l^{-1}). With either decrease or increase from the optimum level, a significant decline in percent plant regeneration was recorded in all the age groups. The comparison of percent plant regeneration in Fig. 1 and Fig. 2 on percent plant regeneration indicated that wounding of cotyledon explants resulted in higher per cent plant regeneration than the unwounded explants owing to better absorption of nutrients on cut surface. Ramirez-Malagon and Ochoa-Alejo, (1996) reported that 9 d old wounded hypocotyl were the best explants for shoot regeneration when inoculated on culture medium without growth regulators. It was observed that very high levels of BAP (9mg l^{-1}) were necessary for maximal shoot regeneration (Hossain *et al.*, 2003). Shivegowda *et al.*, (2003) reported that *in vitro* regeneration was greatly influenced by the cytokinins used. Different combinations of BAP along with IAA, or kinetin also have been reported to induce plant regeneration (Agrawal *et al.*, 1989). Gupta *et al.*, (1998) also reported that BAP 3 mg/l and IAA 1 mg/l were the best shooting supplement.

Number of days taken for shoot regeneration

It was observed from data presented in Fig. 3 and 4 that the number of days taken for shoot bud initiation from wounded and unwounded cotyledon explants varied from 22-28 days. The minimum number of days i.e. 22.66 was taken by the 24 day old wounded cotyledons followed by 18 day old (24.33) on M6 media containing BAP (10mg l^{-1}), kin (5 mg l^{-1}) and IAA (1 mg l^{-1}). Similarly, in unwounded cotyledons as presented in Fig 4 it was observed that minimum number of days to shoot regeneration were taken on M6 media by 18 days old cotyledons i.e. 24.66 followed by 24 day old.

Number of shoots

Data on the average number of shoots from wounded as well as unwounded cotyledons on different media compositions and age groups is presented in Fig 5 and 6, respectively. The maximum number of shoots per explant were recorded on MS media supplemented with BAP (9 mg l^{-1}), kin (2 mg l^{-1}) and IAA (2 mg l^{-1}) in wounded as well as unwounded 24 day explants i.e. 20 and 17 shoots, respectively. But the number of shoots were reduced with either increase or decrease in the concentration of growth regulators. The results showed that 24 day old wounded cotyledons were best explants for generating more number of shoots per explant (20.0) followed by 18 day old wounded cotyledons (18.0) and 24 day old unwounded cotyledon explants i.e. 17.0 shoots per explant. Chilli unlike solanaceous species is considered recalcitrant to regeneration especially at the shoot elongation stage and is the main obstacle in obtaining normal plants. In chilli micropropagation, auxins like IAA in combination with cytokinins like BAP and Kinetin was extensively used for shoot regeneration. The ratio of BAP and IAA i.e. high BAP/low IAA, low BAP/ low IAA and low IAA alone has been reported necessary for normal shoot induction, shoot elongation and optimum rooting, respectively (Phillips and Hubstenberger, 1985). Withdrawal of IAA from the

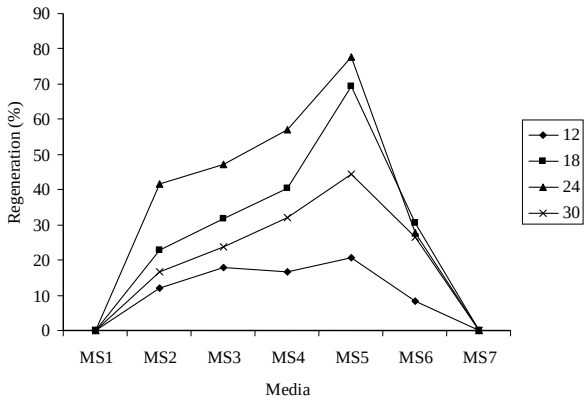

Figure 1. Per cent shoot regeneration from wounded cotyledons as affected by ages of explants and combination and concentration of growth regulators

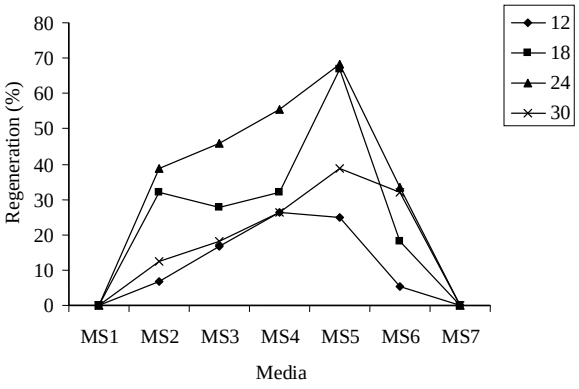

Figure 2. Per cent shoot regeneration from unwounded cotyledons as affected by ages of explants and combination and concentration of growth regulators

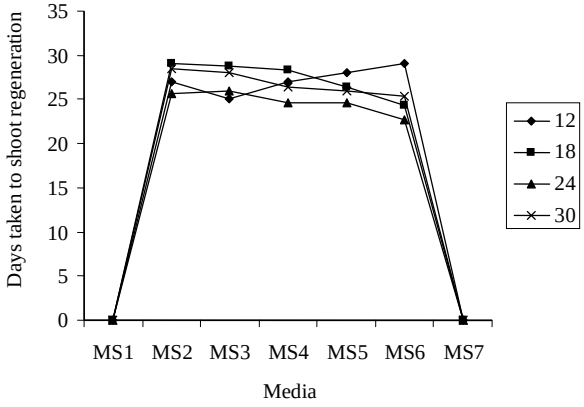

Figure 3. Number of days taken to shoot regeneration from wounded cotyledons as affected by ages of explants and combination and concentration of growth regulators

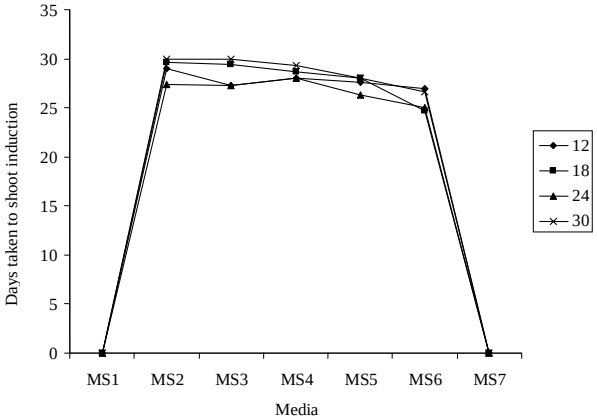

Figure 4. Number of days taken to shoot regeneration from unwounded cotyledons as affected by ages of explants and combination and concentration of growth regulators

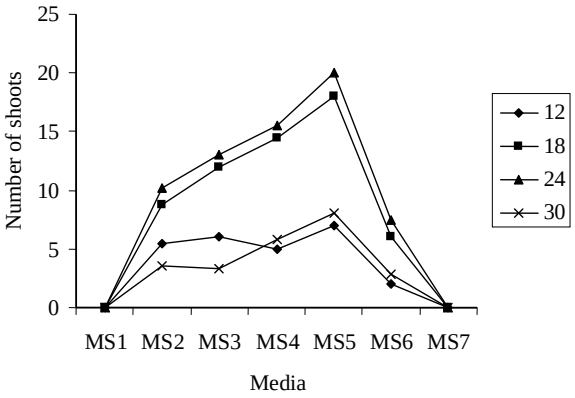

Figure 5 . Number of shoots regenerated from wounded cotyledons as affected by ages of explants and combination and concentration of growth regulators

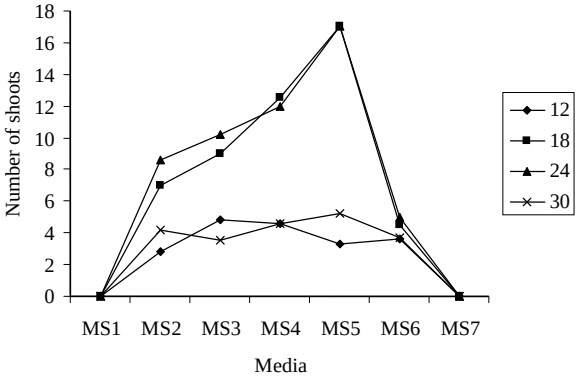

Figure 6 . Number of shoots regenerated from unwounded cotyledons as affected by ages of explants and combination and concentration of growth regulators

Table1: Different concentrations and combinations of BAP, kinetin and IAA used for the shoot regeneration in chilli

Medium	Abbreviation
MS + BAP 1mg/l +Kin 1 mg/l +IAA0.5 mg/l	MS1
MS + BAP 3 mg/l +Kin 2mg/l +IAA1mg/l	MS2
MS + BAP 6mg/l + Kin 4mg/l +IAA 1mg/l	MS3
MS + BAP 7.5 mg/l +Kin 3 mg/l +IAA 1mg/l	MS4
MS + BAP 9 mg/l +Kin 2mg/l +IAA 1mg/l	MS5
MS + BAP 10 mg/l +Kin 5mg/l +IAA 1mg/l	MS6
MS + BAP 10 mg/l +Kin 7.5 mg/l +IAA 1 mg/l	MS7

regeneration medium resulted in an increase in the number of shoots but shoot buds were small (Agrawal *et al* 1989).

Rooting and acclimatization

Shoots thus obtained were isolated aseptically and placed on the rooting media. The percent rooting was 100 percent when *in vitro* raised shoots were cultured on ½ strength MS medium supplemented with IBA (2mg/l) for one week and subsequently transferred to the ½ MS hormone free medium and took two weeks for root initiation. Simultaneous shoot elongation and root formation took place within 10-15 days of culturing on the rooting media. Root system was fully developed within 20-25 days. The roots were well developed and healthy in appearance. Our observations are consistent with the earlier finding in which IBA was successfully employed for rooting in *Capsicum* (Agrawal *et al*. 1988). Shivegowda *et al* (2002) reported that IBA was superior to IAA for rooting of shoots in chilli. Ramirez-Malagon R and Ochoa-Alejo N (1996) reported that rooting was achieved by giving IBA pulses of 60 mg/L for 3 h to shoots followed by sub-culturing on MS medium without growth regulators. Christopher & Rajam (1996) reported that rooting of regenerated shoots was achieved on 5.7 µM IAA containing medium, and the rooting response was better from shoots induced on medium fortified with 5.7 µM IAA plus 13.3 µM BA. Phillips and Hubstenberger reported (1985) that MS media containing 0.05 mg/1 each of IAA and BA promoted shoot elongation and rooting of some explant sources, while 0.05-4 mg/1 IAA with 10-50 mg/1 BA promoted adventitious shoot bud formation.

Since the micropropagated plants are raised in the most congenial environmental conditions, hardening is imperative to ensure the survival of the micropropagated plants upon transfer to soil under the natural conditions Therefore, plantlets were hardened for three days prior to transfer to soil on moist cotton with ½ MS salts. Keeping the potted plants under high humidity during first six days helped a lot to obtain the high survival after planting. Plants having 4-5 well developed leaves performed well after their transfer to soil. The regenerated plants did not show any detectable variation in morphology, exhibited vigorous growth, well developed branches, attained early maturity and displayed the features of male sterility. The experiment replicated for several cycles indicated the reproducibility of the protocol. The tissue culture approach appears highly relevant to multiplication of male sterile lines for further use in hybrid seed production.

सारांश

मिर्च के ms/2 पित्तृ अनुर्वर पंक्ति में विकास उच्च आवृत्ति सामान्य विध के पौध पुनरूत्पादन द्वारा किया गया। पौध पुनरूत्पादन को प्रभावित करने वाले विभिन्न गुणक जैसे एम्स पौध उम्र, बीज पत्र की जख्म और माध्यम संघटक बीज पत्र की ८ दिन उम्र JAA और काइनेटिन, BAP की संघटक और विभिन्न मात्रा के साथ ms माध्यम पूरक पर कृत्रिम और आबादकारी में पौध वृद्धि था। विट्रो में पौध पुनरूत्पादन की आवृति, पौध लेने का दिन और एम्स पौध कली की

संख्या को माध्यम की वृद्धि संघटक और जख्म एम्स पौध की उर्म प्रभावित करता है। 24 दिन की बीज पत्र जख्म को JAA और BAP के साथ उे माध्यम पूरक पर उच्च पौध पुनरूत्पादन (77.77%) न्यूनतम पौध पुनरूत्पादन दिन के लिए अंकित किया जिसमें इस स्तर से वृद्धि, एव न्यूनतम के साथ सार्थक था। कली जब एक सप्ताह के लिए IBA के साथ ms माध्यम पर वृद्धक मुक्त माध्यम के पश्चात् स्थानांतरित किया। विषाणु खादः रेतः मिट्टी (1:1:1) को ग्लास घर स्थिति में स्थानान्तरित कर दिया गया।

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