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TITLE: DEVELOPMENT OF AN EFFICIENT PROTOCOL FOR *IN VITRO* REGENERATION OF CAPSICUM (*CAPSICUM ANNUUM* L.) FROM NODAL EXPLANTS

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Abstract:

The successful implementation of any plant biotechnological approach relies heavily on the establishment of a robust plant regeneration system within the target crop. In this study, the focus was on achieving in vitro regeneration utilizing nodal explants obtained from aseptically raised seedlings of Capsicum namely Solan Bharpur. To optimize germination, seeds underwent various treatments and were subsequently decontaminated before being cultured in vitro on half-strength basal MS medium. Notably, seeds soaked in distilled water supplemented with 1 mg/l GA3 for one day prior to sowing exhibited the most significant impact, resulting in maximum germination rates within a relatively short period. Tissue culture responses were found to be influenced by specific combinations of growth regulators utilized. MS medium supplemented with 2mg/L BAP exhibited shoot regeneration within three weeks. The highest number of shoots per explant (4.0), and average shoot length of 4.5cm was observed in MS medium supplemented with 0.5 mg/l BAP along with 1.0 mg/l Kinetin. Subsequent culturing of regenerated shoots on MS media supplemented with 1 mg/l IBA and 1.5mg/L IAA led to 86% rooting. During the hardening phase, where plantlets were transferred to pots containing sterile coco-peat , the survival percentage was notably higher (89.6%).

Key Words: Capsicum, Tissue culture, Micropropagation, Regeneration, Bell pepper

1. Introduction

Bell pepper (*Capsicum annuum* L.) is a globally cultivated solanaceous crop of significant economic importance, with a particularly strong presence in Asia (Tariq *et al.*, 2014). Bell pepper, also known as capsicum, shimla mirch, sweet pepper, green pepper, spanish pepper, and simply pepper, ranks among the top ten most widely cultivated vegetables globally (Penella and Calatayud, 2018). Within the genus Capsicum, it is primarily composed of diploid, self-pollinating species with a chromosome number of 2n=2x=24 (Hattab et al., 2017), and an estimated genome size of 3.5 Gb (Moscone *et al*., 2003).

Capsicum is extensively cultivated in China, Mexico, Turkey, Indonesia, India, Spain, and the United States, covering a total area of 19,90,926 hectares and yielding approximately 3,80,27,164 metric tonnes annually (FAO, 2019). Introduced to India by the British during the 19th century in the Shimla hills (Bukasov, 1930), capsicum now occupies around 33,000 hectares in the country, with a production of 5,14,000 metric tonnes (NHB, 2020). In India, it is commercially grown in various states including Karnataka, Himachal Pradesh, Haryana, Madhya Pradesh, Maharashtra, Uttarakhand, Jammu & Kashmir, and Tamil Nadu (Sreedhara,

2013). Himachal Pradesh stands as the second-largest producer in India, contributing 59,519 metric tonnes from an area of approximately 2,594 hectares (Anonymous, 2019), with major production districts including Solan, Kullu, Shimla, Mandi, Sirmour, Chamba, and Kangra. Capsicum serves multiple culinary purposes, being consumed raw in salads, cooked as a vegetable, used as a spice, and even pickled or processed. Its nutritional profile is rich, boasting vitamins A, B, C, E, and K, alongside antioxidants such as carotenoids, flavonoids, capsaicin, phenolics, and vitamins, contributing to its health benefits (Djian-Caporalino et al., 2007; Rosa *et al.*, 2002). Moreover, capsicum exhibits anti-allergic, anti-inflammatory, anti-cancerous (Navarro et al., 2006), and pain-relieving properties (Vinaya *et al*., 2009), while finding applications in cosmetics and herbal industries (Bosland and Votava, 2003). The production of bell pepper in India falls behind other crop-producing nations due to a multitude of biotic and abiotic factors that impact its cultivation profitability. Biotic stresses, such as diseases caused by fungi, viruses, bacteria, nematodes, and mycoplasmas, present significant challenges (Maxmen, 2013). Key fungal diseases that hinder capsicum productivity comprise Cercospora leaf spots, damping off, anthracnose, Phytophthora leaf blight, Phytophthora fruit rot, powdery mildew, downy mildew, early blight, and fusarium wilt. Among these, Phytophthora leaf blight and fruit rot stand out as the most prevalent and damaging diseases globally. Solan Bharpur, a mid hill variety of bell pepper is selection from cross between California Wonder and Local Solan Red, with more number of fruits per plant, blocky medium sized fruit weighing 50-60g and is tolerant to Phytophthora blight.

Existing strategies for managing Phytophthora blight include cultural practices, fungicide application, biological control, and planting resistant cultivars, although their effectiveness varies. However, continuous use of limited fungicides has led to pathogen insensitivity over time. Additionally, studies indicate that biological control methods may not always provide satisfactory results (Oelke *et al.*, 2003). Therefore, the primary alternative is to develop and cultivate Phytophthora-resistant varieties.

Micropropagation offers a promising alternative to traditional propagation methods. This technique ensures the production of high-quality, disease-free plants in a shorter timeframe. It focuses on generating true-to-type plants, thereby preserving germplasm integrity. Plant tissue culture techniques present a reliable and efficient means for mass propagation of this species.

2. MATERIAL AND METHODS

2.1 Collection of explants

The seeds of genotypes of *Capsicum annuum* L. were procured from the Department of Vegetable Science, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) and used for the study.

2.2 Cleaning and Autoclaving of glassware

Glassware cleaning involved washing with liquid detergent (Teepol), rinsing with distilled water, and drying in a hot air oven at 80-100℃ for 2-5 hours. Autoclaving followed at 121℃ for 15 minutes at 15 psi. Contaminated medium in flasks was autoclaved and discarded, then glassware was washed, dried, and ready for reuse.

2.3 Nutrient medium Preparation

MS medium was prepared with macronutrients, micronutrients, and supplements. Major and minor nutrient stock solutions, along with vitamins, were refrigerated. Fresh plant growth regulator solutions were prepared, with cytokinins dissolved in NaOH and auxins in alcohol. After adjusting the pH, agar was added to solidify the medium, followed by plant growth regulators. The media were sterilized in flasks at 121°C for 15-20 minutes, then cooled for 2-3 days to ensure sterility before use.

2.4 Aseptic manipulations and culture conditions

Consistent environmental conditions are crucial throughout the incubation period. All components were sterilized and stored in a laminar airflow chamber before inoculation. Aseptic manipulations were conducted in this chamber, prepped with 70% ethanol to reduce contamination risk. Autoclaved equipment and items were placed inside, and UV light was activated for 15-20 minutes. Hands were cleaned with soap and water, then sterilized with alcohol before handling. Cultures were maintained at 25±2°C, 16 hours light, 8 hours dark, with 1500-3000 lux light intensity and 60±5% relative humidity.

2.5 Surface sterilization of seeds

The seeds underwent a series of treatments to facilitate germination. Initially, seeds were washed with tween-20 before being subjected to various conditions. This included soaking in distilled water for 1 day at room temperature and at 4°C, as well as soaking in distilled water containing 1mg/l GA3 for one day at both temperatures. Additionally, fresh seeds were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 1 to 3 minutes in a laminar-airflow cabinet, followed by thorough rinsing with sterile double distilled water to remove any residual $HgCl_2$. The surface-sterilized seeds were then aseptically placed onto half-strength solid MS basal medium and kept at 25±2°C under a 16-hour light and 8-hour dark photoperiod to promote seedling growth. After 5-7 days of *in vitro* growth, these seedlings were utilized to obtain explants for regeneration purposes.

2.6 *In vitro* shoot regeneration and multiplication

Nodal segments developed in vitro were utilized for subsequent in vitro shoot regeneration and multiplication. Multiple combinations of BAP and NAA, at varying concentrations, were added to the MS medium for this purpose. Subsequent subculturing was carried out on standardized medium optimized for shoot multiplication, typically at intervals of 2-3 weeks. Throughout the process, observations were meticulously noted, focusing on parameters like the average number of shoots and their length.

2.7 *In vitro* root induction

Healthy shoots, developed in vitro and aged 3-4 weeks, were transferred to MS rooting medium at either half or full strength, enriched with different concentrations of auxins, either independently or in combination. Throughout this period, various parameters such as the average number of days needed for root initiation, root length, number of roots, and rooting percentage were meticulously documented.

2.8 Acclimatization and Hardening of *in vitro* raised plants

In vitro-grown plants were delicately extracted from the flasks by gently pouring water to prevent damage to the root system, followed by thorough washing to eliminate any traces of agar. The roots were treated with a fungicide solution (2% bavistin) for protection. The plants were then shielded with polybags, with holes punctured to maintain adequate humidity levels. Incubation was carried out under a 16-hour light cycle and a temperature of 25 ± 2°C. After three weeks, the polybags were removed, and the survival percentage of the plants was recorded.

2.9 Statistical analysis:

The data recorded for the different parameters were subjected to Completely Randomized Design. The statistical analysis based on mean values per treatment was made using analysis of variance technique for CRD.

3. RESULT AND DISCUSSION

3.1 Surface sterilization of seeds

The impact of surface sterilization using mercuric chloride at various time intervals was investigated. The treatment employing 0.1% mercuric chloride for 1 minute demonstrated the highest effectiveness. This treatment yielded 81.6% of uncontaminated surviving cultures for the nodes subjected to surface sterilization. The statistical analysis revealed the significance of the results at a 5% level of significance.

3.2 In vitro establishment of cultures using seeds as explant

The seeds were utilized for establishing *in vitro* cultures. Following surface sterilization, the seeds were cultured on Basal MS media. In this study, when fresh seeds were directly placed on MS culture medium without any pre-sowing treatment, the germination rates were notably low. However, pre-sowing treatments significantly improved seed germination, with rates up to 95.45%. Soaking seeds in distilled water before sowing positively influenced both the germination percentage and the speed of germination. Furthermore,

soaking seeds in distilled water along with GA3 at a concentration of 1 mg/l for one day prior to sowing had an even more pronounced effect, resulting in maximum germination occurring in the shortest time frame.

3.3 *In vitro* regeneration and shoot multiplication of cultures using nodal explant

MS medium supplemented with 2.0 mg/L BAP, exhibited shoot initiation within three weeks. Proliferated microshoots were further used for *in vitro* shoot multiplication. Tiny clusters (1-2 cm) were separated and cultured on MS medium supplemented with different combinations of PGRs. Maximum number of shoots (4.0) and average shoot length of 4.5 cm was achieved on MS-3 (0.5 mg/L BAP + 1 mg/L kinetin) however minimum number of shoots (1.0) and average shoot length of 1.5 cm was recorded on MS-5 (1mg/L BAP) (Table 1 and Fig.1). Anilkumar and Nair (2004) similarly highlighted the efficacy of combining 31 mM BAP and 4.6 mM kinetin for inducing multiple shoots from shoot-tip explants in Capsicum annuum L. cv. Early California Wonder. Conversely, Christopher and Rajam (1994) noted that maximal shoot proliferation from shoot-tip explants of Capsicum spp. required considerably higher levels of BAP (ranging from 66.6 to 88.8 mM) and kinetin (ranging from 92.9 to 116.2 mM).

Table 1: Effect of MS medium supplemented with different concentrations and combinations of growth regulators for *in vitro* shoot multiplication

S. No.	Medium Code	MS media (mg/L)		Avg. number of shoots per explant	Average shoot length (cm)
		BAP	Kinetin		
1.	MS-1 (Control)	-	-	-	-
2.	MS-2	1.0	0.5	3.0	3.5
3.	MS-3	0.5	1.0	4.0	4.5
4.	MS-4	0.5	0.25	1.0	2.5
5.	MS-5	1.0	-	1.0	1.5

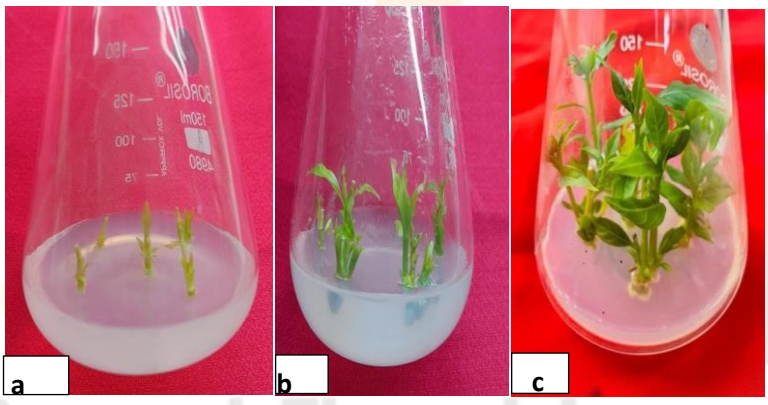

Fig. 1 *In vitro* subculturing and multiplication on MS + 0.5 mg/L BAPand 1 mg/L kinetin

a) **Subculturing of *in vitro* raised microshoots developed from nodal explants**
b) ***In vitro* elongation of microshoots (1 week)**
c) ***In vitro* elongated shoots (3 weeks)**

3.4 In vitro root induction

Fully developed healthy shoots were excised and placed on both full and half strength MS medium supplemented with various combinations and concentrations of auxins. Root initiation was observed approximately 12-20 days after the transfer of shoots to the rooting medium, with complete root system development taking 25-30 days. The most favorable outcomes were achieved with the MS-6 medium,

manifesting within two weeks, yielding an 7 cm root length and 7 roots per shoot (Table 2 and Fig.2) . Comparable findings were also documented by Christopher and Rajam (1994) and Szasz et al. (1995). where they used IAA and IBA combinations for rooting media Additionally, Husain et al. (1999) noted that NAA exhibited greater efficacy in promoting rhizogenesis of the regenerated shoots.

Table 2: Effect of auxins (IAA, IBA, NAA) for *in vitro* root induction from multiplied shoots of Capsicum annuum L.

S. No.	Medium code	Medium strength	MS media (mg/L)				Time taken for initiation (weeks)	Root length (cm)	No. of roots	Rooting percentage (%)
			NAA	IBA	IAA	AC (g/L)				
1.	MS-1(Control)	Half	-	-	-	-	-	-	-	-
2.	MS-2	Full	-	1.0	1.0	-	5	3	3	35 (11.66)
3.	MS-3	Half	1.0	-	-	-	-	-	-	-
4.	MS-4	Half	2.0	-	-	-	-	-	-	-
5.	MS-5	Half	-	2.0	-	-	-	-	-	-
6.	**MS-6**	**Half**	**-**	**1.0**	**1.5**	**1.0**	**2**	**7**	**7**	**86 (18.84)**
7.	MS-7	Half	1.0	1.0	-	-	2	5.5	5	82 (16.82)
8.	MS-8	Half	-	2.0	-	-	-	-	-	-
9.	MS-9	Half	-	1.5	1.5	1.0	3	5	3	56.17(13.70)
	$CD_{0.05}$									**3.28**

values in parenthesis are arc sine transformed value

C.D = Critical Difference

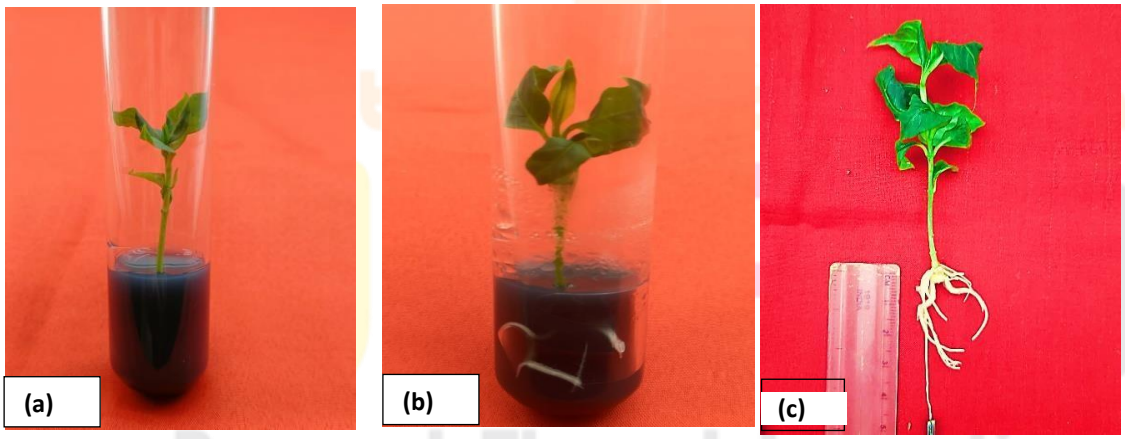

Fig. 2 *In vitro* rooting on ½ MS + 1.0mg/L IBA + 1.5 mg/L IAA + 1g/L AC

a) Inoculation of microshoots on rooting media
b) *In vitro* root induction (2 weeks)
c) *In vitro* raised plantlet (5 weeks)

3.5 Acclimatization and Hardening of *in vitro* raised plants

Acclimatization and hardening of *in vitro*-raised plants are crucial for their survival and successful adaptation to external environmental conditions. This process ensures the success of plant tissue culture experiments. Once rooting is achieved, the plantlets are transplanted into various potting mixtures. Subsequently, the plants are shielded with polybags, with perforations made to maintain adequate humidity levels. Incubation is

carried out under a 16-hour light cycle and temperatures ranging from 25°C to 30°C. Optimal results were observed with cocopeat as the potting mixture, boasting an impressive 89.6% survival rate.

Conclusion

In this study, shoot regeneration was successfully achieved utilizing both seed and nodal explants. The regenerated shoots originating from nodal segments were further multiplied. The utilization of standardized concentrations and combinations of plant growth regulators for plantlet development holds promise for the development of an efficient protocol conducive to large-scale multiplication of this plant species.

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