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PROTOCOL OPTIMIZATION FOR MICRO-PROPAGATION OF *GREEN PEPPER (CAPSICUM ANNUM* L.) CULTIVATED IN ETHIOPIA

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ABSTRACT

Capsicum annuum L (Green Pepper) is one of the most important types of crop plant cultivated all over the world, and particularly in Ethiopia. A successful protocol of *in vitro* plant regeneration has been performed using direct shoot bud induction of the explants of this plant. This study was aiming at developing micro-propagation protocol for green pepper genotype growing in Ethiopia. The best sprouting material for our explants by planting it at three different materials like, sand, manure, and mixture of manure and sand has been well assessed. On MS medium containing 6mg/l 6-benzylaminopurine and 1mg/L indole-3-butyric acid has been recorded relatively highest performance for the regeneration of cotyledons. But propagation for the rest of explants like root, shoot tip and hypocotyls was very poor rather all these explants developed into unorganized mass of cells (calli) after allowing them to be cultured for 12 days. Based on the analysis of the study, only in the cotyledons the direct induction that arose around the cut end of the explants has been revealed. It was on MS medium containing 1mg/L BAP+0.5mg/L indole-3-butyric acid, the enlargement of shoot buds was observed. Once they become self efficient, the plants with good root development were allowed to be shifted into the greenhouse conditions where they further developed, more efficient and matured enough with the estimated survival rate of nearly 85%. With this, the study has confidently concluded that the protocol developed can be used for efficient and successful mass propagation of Ethiopian *Capsicum annuum* L.

KEYWORDS: Green pepper; Mass propagation; Explants; Regeneration; Calli; MS Medium.

INTRODUCTION

Capsicum annum L. are among the classes of fruit plants which belong to the genus *Capsicum* of Solanaceae family (Nightshade). This genus comprises of about twenty five (25) and five (5) wild and domesticated species, respectively (Sanatombi and Sharma, 2007). It is one of the most exploited and economically important plant species of the genus in Ethiopia and all over the world too (Verma *et al*., 2013). It involves both the mild and pungent types of fruits. Its production and multiplication predominantly relies on the conventional system. But this means production and multiplication has certain drawbacks like short survival duration, very poor rate of germination, and high vulnerability to various types of diseases. Moreover, *Capsicum annum* L. is sensitive to different diseases causing microorganisms and pests of which fungi, bacteria viruses and nematodes are the most prevalent. Not only diseases but extremity in climate conditions, especially temperature extremes are also limiting factors for its production (Christopher and Rajam, 1994; Agrawal *et al*., 1988). More reliable micro-propagation approaches for mass production are critically needed, in order the commercial cultivars of these species to be improved and met the alarmingly increasing demands for the crops (Christopher and Rajam, 1994; Agrawal *et al*., 1988).

Plant tissue culture has many applicable techniques of producing large number of propagules within instant period of time which can't be imagined by the natural reproduction process of the plant. Such plant tissue culture related aspects of the green pepper have been thoroughly studied (Phillips and Hubstenberger, 1985; Hussain *et al*., 1999). Many artificial tissue culture techniques for *in vitro* multiplication of this fruit plant have been reported from different parts of the plant, like, shoot tip, (Christopher and Rajam, 1994), rooted/un-rooted hypocotyls, steam, leaf, embryo, root, and cotyledons (Agrawal *et al*., 1989) and induced somatic embryogenesis (Arous *et al*., 2001). However, many of the *in vitro* conditions set for a specific cultivar of the plant proved inappropriate for proper micro-propagation of other cultivars. So, it is necessary to establish reliable proliferation systems for peppers, especially for genotypes developed for commercial use. Moreover, the comparative agronomic performance in terms of valuable traits, such as fruit or seed production, between seed

derived and regenerated plants has not been properly addressed, with only one study reporting the genetic and morphological variation R_0 and R_1 pepper plants regenerated from *in vitro* tissue culture (Guadalupe *et al.*, 2009).

In this study, we have developed an efficient protocol for *in vitro* micro-propagation of an endemic pepper cultivar through direct organogenesis and for subsequent multiplication of the plantlets in the greenhouse (Arous *et al.*, 2001).

MATERIALS AND METHODS

Study Area

The present investigation on "Micro propagation of Green pepper (*Capsicum annum* L)" was carried out in the tissue culture laboratory of the Department of Biotechnology, Adigrat University, Ethiopia, during the academic year of 2016. The details of materials used and methods followed are presented below.

Explant Material and Media Preparation

Green pepper *(Capsicum annum* L) cultivar from local market was taken for investigation and further processing. Samples were kept in mixture of sand and manure for sprouting. The stored sprouted samples were used to get the explants. Then it was followed by the preparation and excision of shoot tip explants of 4–5 cm size from the mother green pepper. Murashige and Skoog (1962) basal medium was used for all the experiments. MS media were prepared from stocks solution. Modification to the medium was done by adding growth regulators and other organic additives.

Preparation of Stocks

Murashige and Skoog (MS) medium was commonly used for all the experiments. The stock solutions were prepared as MS1, MS2, MS3, MS4, and MS5 with 1L for each in separate bottle by filing with double distilled water (DW) to adjust its final volume and were stored in refrigerator at 4^0C. Then stock solutions of 6-benzylamino purine (BAP) were prepared by dissolving first in few drops of 1N NaOH and the volume was made up to the required concentration with double distilled water (dDW).

Preparation and Sterilization of Media

The stock solutions were mixed in required proportion along with growth regulators and 20g/l sucrose. The volume was made up by adding double distilled water. The pH of the medium was adjusted between 5.6-5.8 by using either 0.1 N HCl or NaOH with the help of a digital pH meter. The volume was finally adjusted and required amount of agar (7g/l) was weighed and added into the medium. Agar in the medium was completely melted by gentle heating up to 121^0C and 50ml of medium was poured into each pre sterilized glass culture tubes and plugged with non absorbent cotton wrapped in cheese cloth. The media was autoclaved at 121^0C at 15 lbs/square inch pressure for 20 minutes and then allowed to cool to room temperature and stored in culture rooms until further use.

Surface Sterilization and Inoculation of Explants

Shoot tips, were first washed in water with few drops of liquid soap and detergent (Tween 20) and rinsed with distilled water 3 to 4 times and again they were immersed with NaOCl for 25 min and 70% ethanol for 1 min and then rinsed with distilled water (DW) for 2-3 times. The final surface sterilization was done with distilled water for 3-4 times in the laminar air flow cabinet. Then sterilized explants were inoculated in test tubes containing the media. The cut ends of explants were kept in such a way so as to have maximum contact with the medium.

Transfer area and Maintenance of Aseptic Conditions

All the aseptic manipulations such as surface disinfection of explants, preparation and inoculation of explants were carried out in the laminar air flow cabinet. The working table of laminar air flow cabinet and spirit lamp were sterilized by swabbing with absolute alcohol. All the required materials like media, spirit lamp, lighter, glass ware etc. were transferred on to the clean laminar air flow. The UV light was switched on for half an hour to achieve aseptic environment inside the cabinet where all manipulations were conducted.

Experimental Details

Experiment – I: Standardize the source of explants for micropropagation.
Test "t"tast
Design CRD
Replications: 4
Number of explants used/treatment: 20
Treatment details
T1 – Shoot tip

Experiment – II: Standardize the sterilization procedure for different explants
Test "t"tast
Design CRD
Replications: 4
Number of explants used/treatment: 20
Treatment details
T1 – Sodium hypochlorite (0.5%) 15 min
T2– $HgCl_2$ (0.1%) 15 min

Experiment–III: Standardize the PGR for shoot (growth) multiplication
Test "t"tast
Design CRD
Replications: 4
Number of explants used/treatment: 20
Treatment details
T1 – MS
T2 – MS + BAP 0.5 mg/l
T3 – MS + BAP 1.0 mg/l
T4 – MS + BAP 1.5 mg/l
T5 – MS + BAP 2.0 mg/l

Data Collection

Explants free from Contamination

After inoculation of explants in test tubes, it was ensured to free from fungus, bacteria, browning, but there was contamination of media was recorded.

Number of days taken for Sprouting

The number of days taken to show initial differentiation of shoot from the date of inoculation of different explants was recorded and was expressed as mean number of days.

Percent survival of explants

The number of explants survived and total number of explants inoculated was recorded and converted into percent (%).

Number of shoots produced per explants

Shoot was counted from explants and expressed as shoot per explant.

Number of days taken for initiation of shoots

Number of days taken to show initial differentiation of shoot after 18 days of inoculation was recorded.

Mean length of shoots

The shoot length was measured from base to the tip of the plantlet at the time of subculture and the average length was expressed in centimeters (cm).

Statistical Analysis

The experimental data relating to contamination percentage, percent survival of explant, mean number of shoot and mean length of shoot were collected and analyzed under CRD. The data were subjected to analysis of variance test (ANOVA). Critical difference values were tabulated at one percent (1%) probability wherever**'t'** test found significant. The experimental data relating to explant type were analyzed under**'t'** test.

RESULTS

The results obtained in the present investigation on "micro-propagation of green pepper has been presented under the following headings.

- Standardization of the sprouting material and types of explants for micro-propagation.
- Standardization of the sterilization procedure for different explants.
- Standardization of the growth regulators for shoot growth.

Standardization of the Sprouting Material and Types of Explants for Micro-propagation

Number of days taken for sprouting

The minimum time for sprouting was taken by shoot tip explant in sand and compost to show primordial emergence are presented in the following table.

Table 1: The influence of material on sprouting of explant (shoot) was illustrated as follow.

S. No	Material	No. of Days
1	Sand	9
2	Manure	6
3	Sand and Manure	5
''t'calc	_	1.3
$'t'_{n-1\ \alpha}$	_	1.4
Inference	_	NS

Key1: NS= non significant

Where, n= number of sample '
t'calc. = calculated t value
't' $_{(n-1)}$ α ='t' value from t table at confidence α interval
α =level of confidence (0.01)
n_1 =degree of freedom
If t'calc.>'t' $_{(n-1)}$ α the data is significant
If t'calc <'t' $_{(n-1)}$ α the data is non-significant

Percent Survival of Explants

There was significant difference between the treatments for percent survival of explants.

Table 2: Percent (%) survival of explants.

Group	% Survival of Explant (15 DAT)
A	65% (0.65)
B	78% (0.78)
C	23% (0.23)
D	64% (0.64)
E	56% (0.56)
F	71% (0.71)
t'_{calc}	1.56
$'t_{(n-1)}\alpha$	2.6
Inference	NS

Key2: DAT: day after transfer
NS= non significant

Mean Number of Shoot Produced Per Explants

Significant difference existed among the different level of cytokine growth regulator concentration for number of shoots formed. The shoot tip explant produced the highest number of shoots (6), after the primordial emergence, followed by (4) number of shoots and minimum of (2).

Standardization of the sterilization procedure for different explants

The explants of shoot tip treated with mercuric chloride and sodium hypochlorite for varying periods of time at different concentrations in order to establish maximum contaminant free cultures. The results were presented in the table below.

Table 3: Effect of surface disinfectants on percent (%) contamination and number of healthy cultured established in green pepper explants.

S. No.	Treatments	Exposure time (min)	No. of explants inoculated	No. of explants contaminated	No. of healthy Cultures established
1	Sodium hypochlorite (0.5%)	15	12	8	4
2	$HgCl_2$ (0.1%)	15	12	7	5
t'_{calcu} =		1.67	2.6	3.56	1.38
$t'_{(n-1)}\alpha$		1.2	1.7	4.7	2.4
Inference		S	S	NS	NS

Key 3: NS= non significant
S = Significant

Standardization of the Growth Regulators for Shoot Growth

Number of Days Taken for Initiation of Shoot

There was significant difference with respect to time taken for initiation of shoot growth with respect to its concentration. Time taken for the shoot initiation was minimum (3 days) in 2 mg/l BAP, while it was maximum (6 days) in 0.5 mg/l BAP in shoot tip explant.

Number of Shoots Produced Per Explant

There was significant difference between cytokine growth regulators at different level of concentration for shoot tip explants with respect to the number of shoots produced. The maximum numbers of shoots were produced in shoot tip explant with 2mg/l BAP followed by 1mg/lBA and minimum no of shoot in the control media.

Mean length of shoots

Shoots in media with BAP showed increased shoot length with respect to its concentration. The maximum (8.7cm) and minimum (4.5 cm) shoot length were observed. Increase in the cytokinin concentration in media increase the shoot length. Where as in control media shoot length is nothing that much as compared to media supplemented with BAP.

Table 4: Growth parameters of shoots as influenced by cytokinins in green pepper (shoot tip).

S. No.	Treatment (mg/l)	No. of days taken for shoot initiation	No. of Shoots	Mean length of shoots (cm)
1	MS	-	1	4.5
2	MS + 0.5 BAP	6	2	5
3	MS + 1.0 BAP	5	4	6
4	MS + 1.5 BAP	4	5	8
5	MS + 2.0 BAP	3	6	8.7
't'calc	_	1.56	4.87	3.54
$'t'_{(n-1)}\alpha$	_	2.34	3.12	1.9
Inference	_	NS	S	S

Key 4: NS=non significant
S=significance

DISCUSSION

The present investigation was undertaken to standardize or optimize the protocols for culture establishment, multiple shoot production, *in vitro* rooting for micro-propagation of green pepper were assessed. The function of culture establishment is to disinfect the explant, establish explant in culture media, and stabilize the culture media and the explant for multiple shoot production (Mc Cown, 1986).

Standardization of the Sprouting Material and types of Explants for Micro-propagation

The type of organs or explants chosen affects the successful establishment of the cultures and their subsequent growth. Not all the tissues or organs of a plant are equally capable of exhibiting morphogenesis (Hartmann *et al.*, 1997).

In the present study, to identify a suitable explant for *in vitro* propagation of green pepper, different explants were tried. Among the various explants, shoot tips gave the quickest response for initial growth and the highest number of multiple shoots at higher level of cytokine growth regulator. On the other hand, Shoot took time for the regeneration at lower level of cytokine growth regulator concentration. This difference in response, among the different level of cytokine growth regulator might be due to difference in physiological state of the explants for exposure of plant growth regulator hormone (Sreelatha *et al.*, 1998). This may also be due to the fact that, the shoot tip has meristematic region where cell division and differentiation occurs at optimum level of growth hormone so fast (Hartmann *et al.*, 1997). Murashige (1978) made a similar observation and reported that shoot tips are highly regenerative when

they are exposed at optimum level of growth regulator. The highest numbers of multiple shoots were produced by shoot tip explant. This may be due to excised apex when placed on the medium with high inorganic nutrient salt and optimum level of growth regulator. In the present study, the shoot tip gave maximum multiple shoots at optimum level of cytokine growth regulator and survival percentage which is in line with the findings of Malmug *et al.* (1991), Mukund (1998), Balakrishnamurthy and Rangaswamy (1992) in banana.

Standardization of the Sterilization Procedure for Different Explants

In vitro propogation involves culturing explants under aseptic conditions in which surface sterilization or disinfection is one of the important prerequisites for successful micropropagation. Removing contaminants from the surface of the organ/explant is of prime concern (Hartmann *et al.,* 1997). The contamination of explants may be due to fungi, bacteria, moulds, yeasts etc., present on the surface or lodged in the cracks, scales etc. General disinfection procedures have been given by various workers for plant tissues (Krikorian, 1982). Disinfection requires the use of chemicals that are toxic to microorganism's but non-toxic to plant materials. Tissue culture became possible with the use of convenient and effective disinfectants such as ethanol, sodium hypochlorite, mercuric chloride, calcium hypochlorite and others (Krikorian, 1982).

The current investigation was on the effect of surface sterilants on reducing contamination rate and percent of healthy cultured plants. It showed that $HgCl_2$ was better sterilant than NaOCl in reducing contamination rate. This was because the most useful radical in $HgCl_2$ is probably the chlorite, commonly present as bichloride of mercury. Mercuric chloride is extremely poisonous due to high bleaching action of two chloride atoms and also mercuric ions which combines strongly with protein causing death of organism (Pauling, 1955). Even though NaOCl consists of chlorine atom, its bleaching and disinfectant action is due to the slow decomposition of the salt to produce oxygen (Rahman *et al.* 2004).

The highest numbers of aseptic culture was obtained with $HgCl_2$ at 0.1 per cent for 15 minutes. Different authors have reported, differential response from horticultural crop, to get contaminant free cultures using different durations. Raju *et al.* (2005) got the results using 0.1 percent $HgCl_2$ for 15 minutes and Rahman *et al.* (2004) used 0.1 percent $HgCl_2$ for 14 minutes to establish aseptic cultures in laboratory. These findings are conforming to the results obtained by Nadagouda *et al.* (1983) in cardamom. The higher concentration of $HgCl_2$ at 0.1 percent for 15 minutes observed more contamination and also death of the explants. This may be due to the high bleaching activity of chlorine which killed the cells. The lowest aseptic cultures were obtained with NaOCl 0.5%. This was because of reduced effectiveness of chemicals at lower concentrations. Optimal concentration of chemical and duration of chemical in the present Investigation was $HgCl_2$ at 0.1 percent for 15 minutes with respect to low contamination rate, which is in accordance with the findings of Raju *et al.* (2005).

Standardization of the Growth Regulators for Shoot Growth

The results revealed that, the multiple shoot formation was more in high concentration of BAP as compared to low concentration of BAP. This was in confirmation with the results of Wong (1986) who observed that BAP is the cytokinin of choice for induction of shoot bud proliferation at high concentration *in vitro* and BAP has been found to be superior at optimum/higher concentration in banana.

In the present study, the less number of days for initiation and the highest number of multiple shoots were observed in 2mg/l BAP supplemented media. This was in confirmation with the results of Dipti *et al.* (2005), who reported that the highest number of multiple shoots in media supplemented with 2 mg/l BAP in shoot tip and 3 mg/l BAP in green pepper explants, in turmeric proved its superiority over at its lower concentration by producing more number of multiple shoots. BAP at 3 mg/l was most beneficial for proliferation in turmeric (Balachandran *et al.,* 1990). Similarly, Winnar and Winnar (1981) reported that BAP 1mg/l was most useful for development of multiple shoots. These findings are in conformity with the research result of Ali *et al.* (2004).

The results of the study revealed that the number of shoots increased and the mean length of shoots increased as the concentration of cytokinins increased. Media with the highest cytokinin concentration showed the maximum number of multiple shoots and higher length of shoots. This may be due to the fact that suppression of apical dominance leads to the production of more number of multiple shoots and reduced shoot length (Balachandran *et al.*, 1990).

Protocol for Micro-propagation of Green Pepper

Based on the results, a protocol for micro-propagation of green pepper has been summarized in the following way. First of all, shoot tips of 4-5 cm should be isolated. Then the isolated explants are to be washed with distilled water and liquid soap. They need to be treated with 0.1 percent HgCl2 for 15 minutes and then washed 3-4 times with sterile distilled water in laminar air flow cabinet. After sterilization, explants need to be cultured on MS medium for 3 weeks. Then these shoots may be cultured on basal medium with 2mg/l BAP for shoot multiplication. After 2-3 subcultures micro shoots may be placed on MS medium containing 1 mg/l IBA for rooting. Finally, these rooted plantlets have to be hardened in green house.

CONCLUSION AND RECOMMENDATION

We have developed optimum protocol for an efficient regeneration from shoot proliferation of Ethiopian green pepper by generating best surface sterilizing prouder in aseptic condition, and by combining cytokine growth regulator at different concentration with other essential nutrient, and also by selecting suitable material for sprouting. Plant cell are totipotent, hence they have capacity to divide, grow and differentiate in to whole organ (like, into shoot, root, stem, node and also into flower) when they are grow in appropriate nutrient media.

Aseptic condition and subculturing during plant tissue culture is highly recommended for efficient result. Moreover, plant tissue culture is the back bone for the development of agricultural product, industrial product, medical and pharmaceutical drug production, and for other relevant aspects. Therefore, we suggest this technology is highly important for developing country, especially for Ethiopia through which there is no well developed plant tissue culture (PTC) company to produce /provide disease free plant. Ethiopian farmer can't get large product of agrarian crop which are free from disease. As a result, the government and plant tissue culture expert must take responsibility to solve this problem by developing well organized PTC Company, which is aseptic and relevant for the generation of pure and disease agrarian crop. By taking this in to account, we all should be get up to apply such relevant and amazing green technology in to application.

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