

Optimization of Regeneration Protocols of Chilies In Local Cultivars

Mahnoor Khan¹, Ummul Buneen¹, Syed Naem Sajid^{1*}, Maliha Nawaz¹,
Haseeb Harron², Imtiaz Hussain¹, Abdul Haseeb³, Muhammad Hassan Taj¹,
Faisal Nadeem¹, Muhammad Kamran Rafique¹ and Hamza Akram¹

¹Centre of Agricultural Biochemistry & Biotechnology (CABB), University of Agriculture,
Faisalabad, Pakistan.

²National Institute of Food Science and Technology, University of Agriculture Faisalabad, Pakistan.

³Department of Plant breeding and Genetics, University of Agriculture, Faisalabad, Pakistan.

<http://dx.doi.org/10.13005/bbra/2819>

(Received: 22 February 2020; accepted: 06 April 2020)

Chili (*Capsicum annum*) is the third most important horticultural crop of family *Solanaceae* after potatoes and onions. This plant belongs to tropical and sub-tropical region. Warm and humid climate is required during growing stage but for the maturity of the chili plant dry weather is required. An efficient plant regeneration protocol is necessary to improve genetic makeup of chili crops. Chili pepper is sensitive to regeneration system in contrast to other solanaceous species which show great ability for regeneration. Additionally chili peppers are extremely vulnerable to viral and fungal pathogens which cause significant damage to these crops. Low germination rate of seeds and short viability time period are also some major drawbacks of chilies. As a consequence, tissue culture techniques are used due to which large number of disease free and genetically pure chili plants can be produced. The quality of crops can be improved by developing resistance against diseases. A study was accompanied in which explants of roots, stem, leaves and cotyledons of chili were used for regeneration on MS basal medium complemented with different concentrations of auxins and cytokinins. Callus induction from roots, stem, leaves and cotyledons explants under *in vitro* conditions was developed on MS medium supplemented with different plant hormones such as IAA, BAP and 2,4-D. After the induction of shoots and roots on MS medium supplemented with IAA and BAP, the rooted plants were hardened in the growth room for the development of complete plant, which gave a basis for the formation of transgenic plants.

Keywords: Chili (*Capsicum annum*), IAA, BAP and 2,4-D, *Capsicum*, Caribbean, Tracheophyta.

Chili

Chili is an important horticultural crop of the world, commonly known as pepper, sweet pepper or bell pepper. Chilies belong to genus *Capsicum* of the family *Solanaceae* which is also called night shade. The plants of the *Capsicum* genus are represented by the terms; chili, chile, chili, paprika, aji, pepper and capsicum (DeWitt

and Bosland, 1993). This plant is originated from Central and South America as a wild crop around 7500BC (Grubben and El Tahir 2004). The term “bell pepper” usually refers to a non-pungent and sweet chili pepper type whereas term “chili pepper” is used to refer to a pungent chili variety. This essential item of kitchen is used daily in one or the other form as a condiment, adding flavour,

*Corresponding author E-mail: snomi432@gmail.com



colour and pungency to numerous foodstuffs. The genus *Capsicum* contains more than 25 species, out of which only 5 species namely *C. annuum* L., *C. pubescens*, *C. baccatum*, *C. frutescens* and *C. chinense* are domesticated (Kothari *et al.*, 2010). Amongst these, *Capsicum annuum* L. is most cultivable all over the world and is the most vital economical species. Two cultivars of *Capsicum annuum* L. known as 'Meiteimorok' and 'Haomorok' locally are main food crops of Manipur. The fruits of 'Meiteimorok' are slightly pungent and are widely used in dried and fresh forms equally, whereas fruits of 'Haomorok' are mild, and are used as fresh green chillies for consumption.

The word *Capsicum* comes from "Kapsimo" which means to bite (Basu, De *et al.*, 2003). Christopher Columbus was the first European who discovered it in the Caribbean. On his trip to America Columbus tastes a plant whose perception was similar to black pepper. He took that plant back to Europe and then was presented to Africa and Asia. Carl Linnaeus named this plant as *Capsicum* afterwards (Valadez-Bustos, Aguilera-Santacruz., 2009).

Different intensities of pungent flavour are present in every kind of chili. That pungency is because of a chemical which is known as Capsaicinoids. Capsaicinoids are located in the placenta of the fruit only. There is a wide variety of nutrients present in the capsaicinoids which expose anti-oxidant and anti-carcinogenic properties (Khan, Asghar *et al.*, 2014). There has been a significant increase in chili crop production level since the late 1990s. In 2007-2008, worldwide chili production was projected as 20.98 lac tons. China, India, Mexico, Peru, Ethiopia, Pakistan, Myanmar, Vietnam, Bangladesh and Ghana are considered as the highest chili producing countries. Chili production in Pakistan from year 2010-16 is given in table 1.2.

The substances that determine pungency (spicy heat) of chili peppers are capsaicin and numerous associated chemicals, collectively known as capsaicinoids. Capsaicinoids are bioactive particles which are linked with food and medicinal sciences. More the content of capsaicinoids more will be its anti-oxidant properties and higher will be the pungency as indicated by *in vitro* analysis.

Capsaicinoids are mostly consumed as naturally occurring spiciness generating constituents of *Capsicum*. The concentration of capsaicinoids in chili pepper is 0.1mg/g, in red pepper is 2.5mg/g and in oleoresin red pepper is 60mg/g. Capsaicinoids are alkaloids in nature. Naturally occurring capsaicin (69%), dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homodihydrocapsaicin (1%) and homocapsaicin (1%) are five main types of capsaicinoids (Kosuge and Furuta 1970). Among these, capsaicin and dihydrocapsaicin are found in abundance in chili peppers, constituting about 90% of the entire capsaicinoid, whereas the remaining types of capsaicinoids contribute a little in overall capsaicinoid content and spiciness of the fruit (Montgomery, 1999). Capsaicinoids can be extracted in numerous ways; however the yield differs with chili variety and the conditions of extraction/processing. For the extraction of oleoresins, supercritical fluid system has been used because this system is fast, simple and inexpensive as compared to traditional methods (Maceration, Soxhlet). Chili pepper has been domesticated in all over the world as a vegetable and colouring agent. With the increase in population the enhancing needs of the world can't be satisfied just by growing chilies by the means of seeds. It requires mass production of the cultivar in relatively short period of time. To overcome this problem tissue culture method arises. The procedure of *in vitro* propagation of explants that allows permissible variation of the edifice is known as tissue culture. This *in vitro* culture has several commercial applications. It is an important constituent of plant biotechnology. In 1902 Gottlieb Haberlandt gave the academic foundation for plant tissue culture on his experiments on culturing the plants under *in vitro* conditions. He, therefore, evidently introduced the idea of totipotency. Regarding his speech and his innovative research, Haberlandt is arguably known as the father of plant tissue culture and apparently it was first presented by (Steward *et al.*, 1958). It provides an efficient mean to regenerate novel plants from genetically engineered cells. The favourable plant thus produced may be readily cloned in cultures under sterilized conditions. Tissue culture is considered an alternative mean for vegetative plant propagation. Regeneration techniques are helpful in maintaining physical

environment and growth conditions as compared to conventional methods (Shibli *et al.*, 1992). Additionally evaluation of biotic and abiotic stresses and regulation of growth parameters can be achieved through tissue culture (Grattan *et al.*, 2002). These advantages describe that few factors such as physiological effects, interaction of hormones and metabolism required for normal plant growth can be completely examined (Baire and Kane, 2011).

In vitro propagation of plants is an acceptable and consistent method to obtain disease free and improved yield crops in short period of time (Ashrafuzzamnet *et al.*, 2009). In vitro plant regeneration is significant in conserving pure genetic material. It is also beneficial as it protects the plants from bacterial and fungal diseases. Due to low production rate conventional breeding method is limited. Micro propagation has several advantages as compared to conventional breeding method as it leads to successful propagation of novel genotypes (Bhagowati and Changkija 2009). Micro propagation provides an improved and healthier source of medicinal and other economically important plants in short time and limited place. Chilies are extremely susceptible to fungal and viral attacks (Evans, Sharp *et al.*, 1983). Tissue culture technique is beneficial as it protects the plants from fungal and viral attacks. Different explants i.e. hypocotyls, cotyledons; protoplast, shoot organogenesis and direct somatic embryogenesis are used to obtain in vitro plant regeneration. Tissue culture technique has several advantages, some of which are:

- Attaining disease free plants.
- Precise environmental conditions.
- Fast proliferation of plants those are difficult to proliferate.
- Somatic hybridization.
- Genetics enhancement of commercial plants.
- Attaining androgenic and gynogenic haploid plants for breeding programmes.
- Verify the availability of plants throughout the year.
- Primary and secondary metabolites are obtained from plants with medicinal effects.
- Preservation of endangered plant types.
- Conservation of genetic material by cryopreservation.

MATERIAL AND METHODS

The research work for the optimization of regeneration protocol of chili using various explants was conducted in Virology Laboratory, Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad during the year 2017-2018.

Plant Profile

Taxonomic classification of *Capsicum annuum* provided by PLANT database of USDA-NRC (<http://plants.usda.gov>) is given below.

Seed Collection

Seeds of Chili (*Capsicum annuum* L. variety SVHA 1182) were obtained from vegetable department of Ayub

Preparation of working Media

For the preparation of 1 liter medium 4.33g/l Murashige and Skoog basal salts mixture was added in an autoclaved beaker containing 800ml autoclaved double distilled (d3H2O) water. With the help of magnetic stirrer it was dissolved completely then 30g/l sucrose was added and distilled water was added to make up the final volume up to the required level. The pH was kept 5.6 to 5.8 by using 5M NaOH or 100% HCl and 8g/l phytigel was added to the MS medium at the end. MS media bottle was autoclaved at a temperature of 121°C under 15 psi pressure for about 20 mins. Then laminar air flow cabinet was sterilized with 10% commercial bleach or 100% ethanol. UV light was turned on for 20 minutes. After 20 minutes UV light was turned off and work was started. Media was allowed to cool and finally poured into petri plates. The plates were left for solidifying and were covered with the help of cling film and stored at 4°C.

Surface Sterilization of Seeds

Seeds were first rinsed with autoclaved distilled water for 2 mins to remove dust particles. Seeds were surface sterilized with 10% bleach in a 15ml falcon tube. The falcon tube was shaken gently by inverting for 5 minutes. Then the bleach was discarded and the seeds were splashed three times with doubled distilled autoclaved water to remove traces of bleach. Then the seeds were placed on autoclaved filter paper and left for drying.

Germination of Seeds

Washed seeds were placed on sterilized filter paper to dry them for 10 mins and transferred

to petri plates in a MS medium. The plates were enfolded with cling film to get rid from contamination. The plates were placed in the growth room at 26+2°C under photoperiod of 16 h light and 8 h dark. The seeds started to germinate after one week.

Selection of explants

In the laminar air flow, petri plates with healthy seedlings were selected to use as explant. Two week old seedlings of stem, leaf, root and cotyledon explants were cut into small pieces of 2-3 mm in length.

Callus Induction

Plant Growth Regulators (PGRs) Combination in Callus Induction Medium

Different combinations and concentrations of PGRs were tested for studying the optimum response for callus. The media and their formulations are provided below.

- 1) CIM1= 5µL IAA+50µL BAP/500ml MS media.
- 2) CIM2=5µL NAA+50µL BAP/500ml MS media.
- 3) CIM3=5µL IAA+50µL 2, 4-D/500ml MS media.

Table 1.2. Chili production in Pakistan

| Years | Production (tons) |
|-------|-------------------|
| 2010 | 306980 |
| 2011 | 305088 |
| 2012 | 307080 |
| 2013 | 300000 |
| 2014 | 290000 |
| 2015 | 277137 |
| 2016 | 265000 |

Table 3.1. Biological classification of *Capsicum annuum*

| | |
|---------------|--------------------------------------|
| Kingdom | Plantae-Plants |
| Subkingdom | Tracheophyta-Vascular plants |
| Superdivision | Spermatophyta-Seed plants |
| Class | Magnoliopsida-Dicotyledons |
| Subclass | Rosidae |
| Order | Solanales |
| Family | Solanaceae |
| Genus | <i>Capsicum</i> |
| Species | <i>Capsicum annuum</i> Lush. - chili |

Callus Induction from *In Vitro* Grown Plants From stem explants

Stem explants were taken from 2 weeks old *in vitro* grown plantlets. With the help of forceps explants were placed on the plates containing solidified callus induction media, and were placed in the dark at 26+2°C to induce callus. Callus initiation was observed from both sides of the stem explants after 10 days. After the development of compact and hard texture callus it was again placed in light. Then the callus was cut into small pieces. These pieces were transferred onto fresh medium to increase the friability of callus. Observations were noted carefully and kept record so that explants can be tracked.

From leaf explants

Leaf explants were taken from 2 weeks old *in vitro* grown plantlets. With the help of forceps explants were placed on the plates containing solidified callus induction media, and were placed in the dark at 26+2°C to induce callus. Callus initiation was observed from both sides of the leaf explants after 10 days. After the development of compact and hard texture callus it was again placed in light. Then the callus was cut into small pieces. These pieces were transferred onto fresh medium to increase the friability of callus. Observations were noted carefully and kept record so that explants can be tracked.

From root explants

Root explants were taken from 2 weeks old *in vitro* grown plantlets. With the help of forceps explants were placed on the plates containing solidified callus induction media, and were placed in the dark at 26+2°C to induce

Table 3.3. MS0 media composition

| Components | Concentration |
|----------------|---------------|
| Sucrose | 30g/L |
| MS salt | 4.33g/L |
| Glycine | 2.0mg/L |
| Nicotinic acid | 0.5mg/L |
| Pyridoxin | 0.5mg/L |
| Thymine HCl | 0.1mg/L |
| Myo-inositol | 100mg/L |
| Agar | 8.0g/L |
| pH | 5.7-5.8 |

callus. Callus initiation was observed from both sides of the root explants after 10 days. After the development of compact and hard texture callus it was again placed in light. Then the callus was cut into small pieces. These pieces were transferred onto fresh medium to increase the friability of callus. Observations were noted carefully and kept record so that explants can be tracked.

From cotyledon explants

Cotyledon explants were taken from 2 weeks old *in vitro* grown plantlets. With the help of forceps explants were placed on the plates containing solidified callus induction media, and were placed in the dark at 26±2°C to induce callus. Callus initiation was observed from both sides of the cotyledon explants after 10 days. After the development of compact and hard texture callus it was again placed in light. Then the callus was cut into small pieces. These pieces were transferred onto fresh medium to increase the friability of callus. Observations were noted carefully and kept record so that explants can be tracked.

Callus maintenance

Actively growing and friable calluses were maintained by transferring subcultures onto a fresh medium after 4 week interval under aseptic conditions.

Shoot Induction

PGRs Combination in shoot induction Medium (SIM)

Different media were tested for studying the optimum response for shoot induction. The media and their formulations are given below.

- 1) SIM1= 5µL IAA+50µL BAP/500ml MS media.
- 2) SIM2=10µL IAA+55µL BAP/500ml MS media.
- 3) SIM3=20µL IAA+60µL BAP/500ml MS media.

Shoot Induction to Callus obtained from *In Vitro* Explants

Shoot development from stem explants derived callus

Callus obtained from *in vitro* derived stem explants were placed on different shoot induction media for shoot development under strict aseptic conditions. Firstly explants were cultured on shoot induction media (SIM). Callus was shifted to next shoot induction media with same concentrations and observations were recorded after each week interval. Special care was taken while transferring callus to get rid from contamination. Callus was sub-cultured after 2 weeks interval.

Shoot development from leaf explants derived callus

Callus obtained from *in vitro* derived leaf explants were placed on different shoot induction media for shoot development under strict aseptic conditions. Firstly explants were cultured on shoot induction media (SIM). Callus was shifted to next shoot induction media with same concentrations and observations were recorded after each week interval. Special care was taken while transferring callus to get rid from contamination. Callus was sub-cultured after 2 weeks interval.

Shoot development from root explants derived callus

Callus obtained from *in vitro* derived root explants were placed on different shoot induction



Fig. 4.1. Surface sterilization of seeds



Fig. 4.2. Seeds washing

media for shoot development under strict aseptic conditions. Firstly explants were cultured on shoot induction media (SIM). Callus was shifted to next shoot induction media with same concentrations and observations were recorded after each week interval. Special care was taken while transferring callus to get rid from contamination. Callus was sub-cultured after 2 weeks interval.

Shoot development from cotyledon explants derived callus

Callus obtained from *in vitro* derived cotyledon explants were placed on different shoot induction media for shoot development under strict aseptic conditions. Firstly explants were cultured on shoot induction media (SIM). Callus was shifted to next shoot induction media with same concentrations and observations were

recorded after each week interval. Special care was taken while transferring callus to get rid from contamination. Callus was sub-cultured after 2 weeks interval.

Root Induction

PGRs Combinations and concentrations in root induction medium

1) RIM1= 10 μ L IAA+50 μ L BAP/500ml MS media.

2) RIM2=20 μ L IAA+55 μ L BAP/500ml MS media.

3) RIM3=30 μ L IAA+60 μ L BAP/500ml MS media.

Root Induction to shoots developed from *in vitro* explants

Root Induction to shoots developed from stem explants

The shoots developed from stem explants were shifted to root induction media (RIM) for root



Fig. 4.3. One week old seed germinating

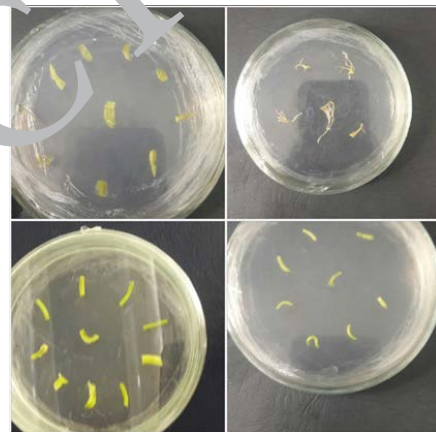


Fig. 4.5. Cuttings of two week old seedlings into small segments of 2-3 mm length



Fig. 4.6. Callus induced in stem explants after 10 days

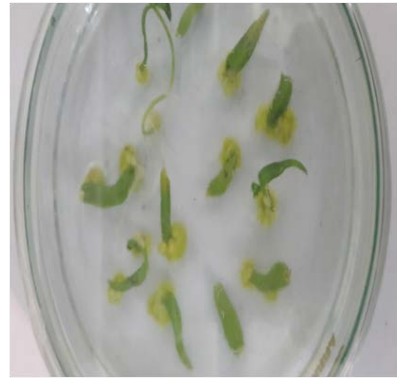


Fig. 4.7. Callus initiation started from leaf explants after 10 day

induction. Root formation was started from stem callus after 2-3 weeks.

Root induction to shoots developed from leaf explants

The shoots developed from leaf explants were shifted to root induction media (RIM) for root induction. Root formation was started from stem callus after 2-3 weeks.

Root induction to shoots developed from root explants

The shoots developed from root explants were shifted to root induction media (RIM) for root induction. Root formation was started from stem callus after 2-3 weeks.

Root induction to shoots developed from cotyledon explants

The shoots developed from cotyledon explants were shifted to root induction media (RIM) for root induction. Root formation was started from stem callus after 2-3 weeks.

Hardening of regenerated plants

Hardening of regenerated plants was the last and most sensitive step of plant tissue culture which required a great care during handling. Rooted plantlets were removed from glass jars and their roots were washed with distilled water. To get rid from fungal infection, roots were dipped in a fungicide solution. Regenerated plants were transferred to pots containing compost for more shoot elongation and healthy root development. Compost contains plant litter, animal manure, sand and soil. It provides large amount of nutrients required by the plantlets. Initially pots were covered with polyethylene bags for one week, and were kept in the growth room at $22 \pm 2^\circ\text{C}$. After one week a hole was created in the polythene bags to make the plantlets resistant from environmental stress.



Fig. 4.8. Callus initiation started from root explants after 10 days.

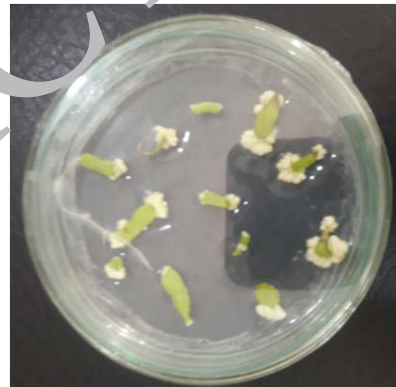


Fig. 4.9. Callus initiation started from cotyledon explants after 10 days.



Fig. 4.10. Shoot Induction from stem callus after 14 days

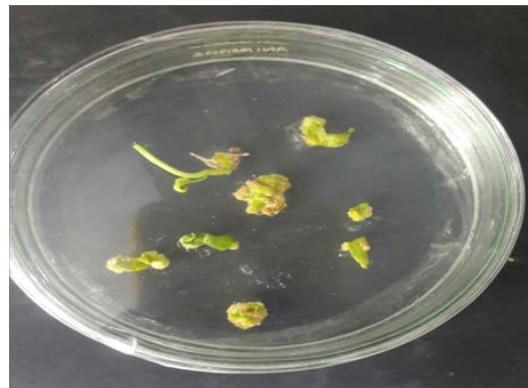


Fig. 4.11. Shoot induction from leaf callus after 14 days

RESULTS

The best levels and combinations of plant hormones (PGRs) for efficient regeneration of *Capsicum annum* L. variety SVHA 1182 by using different parts as explants were investigated.

Seeds Sterilization

Seeds of *Capsicum annum* L. variety SVHA 1182 were sterilized under controlled conditions to get contamination free explants. For the removal of dust particles seeds were thoroughly washed with tap water. Further sterilization was performed by immersing the seeds in 10 % bleach for about 10 minutes and then three times rinsed with autoclaved distilled water for 10-15 minutes until the traces of bleach were gone. This whole work was done in a laminar air flow cabinet by using UV light that gave maximum hygienic and sterilized conditions in working area.

Germination of seeds

After sterilization, seeds were placed in petri plates for germination. Plates containing seeds were placed in the growth room under a 16/8h (light/dark) regime at a temperature of 26±2°C. Seeds started germinating after one week of incubation.

Selection of explants

In the laminar air flow, petri plates with healthy seedlings were selected to use as explant. With the help of blade two week old seedlings of stem, leaf, root and cotyledon explants were cut into small pieces of 2-3 mm in length.

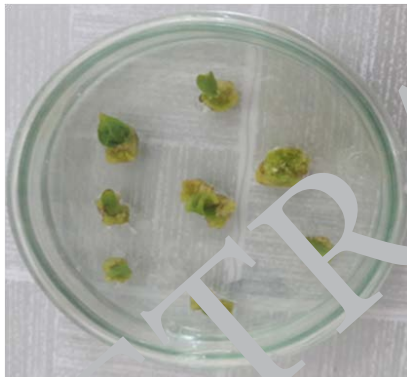


Fig. 4.12. Shoot induction from cotyledon callus



Fig. 4.13. Shoot induction from callus after 3 weeks



Fig. 4.14. Root induction started from callus after 2-3 weeks



Fig. 4.15. Hardening of Regenerated plants covered with polythene bags

Callus Induction

Response of stem explants to callus medium

To induce callus stem explants of *Capsicum annuum* L. were used. For this purpose three types of media were prepared having different concentrations of plant hormones (PGRs).

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 5 μ L NAA+50 μ L BAP/500ml MS media.
- 3) 5 μ L IAA+50 μ L 2, 4-D/500ml MS media.

When stem explants were transformed on these three callus induction media (CIM) maximum callus was obtained on media which have high conc. of BAP and low conc. of IAA. On the other hand, minimum callus was obtained from the other two media. Callus initiation was observed from both sides of the stem explants after 10 days. To get rid of rooting the explants were kept in dark for about 5-6 days. After the development of compact and hard texture callus it was again placed in light. Then the callus was cut into small pieces. When these pieces were transferred onto fresh medium friability of callus was increased.

Response of leaf explants to callus medium

To induce callus leaf explants of *Capsicum annuum* L. were used. For this purpose three types of media were prepared having different concentrations of plant hormones (PGRs).

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 5 μ L NAA+50 μ L BAP/500ml MS media.
- 3) 5 μ L IAA+50 μ L 2, 4-D/500ml MS media.

When leaf explants were transformed on these three callus induction media (CIM) maximum callus was obtained on media which have high conc. of BAP and low conc. of IAA. On the other



Fig. 4.16. Hardening of Regenerated plant

hand, minimum callus was obtained from the other two media. Callus initiation was observed from both sides of the leaf explants after 10 days. To get rid of rooting the explants were kept in dark for about 5-6 days. After the development of compact and hard texture callus it was again placed in light. Then the callus was cut into small pieces. When these pieces were transferred onto fresh medium friability of callus was increased.

Response of root explants to callus medium

To induce callus root explants of *Capsicum annuum* L. were used. For this purpose three types of media were prepared having different concentrations of plant hormones (PGRs).

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 5 μ L NAA+50 μ L BAP/500ml MS media.
- 3) 5 μ L IAA+50 μ L 2, 4-D/500ml MS media.

When root explants were transformed on these three callus induction media (CIM) maximum callus was obtained on media which have high conc. of BAP and low conc. of IAA. On the other hand, minimum callus was obtained from the other two media. Callus initiation was observed from both sides of the root explants after 10 days. To get rid of rooting the explants were kept in dark for about 5-6 days. After the development of compact and hard texture callus it was again placed in light. Then the callus was cut into small pieces. When these pieces were transferred onto fresh medium friability of callus was increased.

Response of cotyledon explants to callus medium

To induce callus cotyledon explants of *Capsicum annuum* L. were used. For this purpose three types of media were prepared having different concentrations of plant hormones (PGRs).

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 5 μ L NAA+50 μ L BAP/500ml MS media.
- 3) 5 μ L IAA+50 μ L 2, 4-D/500ml MS media.

When cotyledon explants were transformed on these three callus induction media (CIM) maximum callus was obtained on media which have high conc. of BAP and low conc. of IAA. On the other hand, minimum callus was obtained from the other two media. Callus initiation was observed from both sides of the cotyledon explants after 10 days. To get rid of rooting the explants were kept in dark for about 5-6 days. After the development of compact and hard texture callus it was again placed in light. Then the callus was cut into small pieces. When these pieces were

transferred onto fresh medium friability of callus was increased.

Shoot induction

Response of stem callus to shoot induction

2 weeks old callus of stem explants was cultured on the shoot induction medium (SIM) supplemented with different concentrations of auxins and cytokinins.

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 10 μ L IAA+55 μ L BAP/500ml MS media.
- 3) 20 μ L IAA+60 μ L BAP/500ml MS media.

Shoot buds started to initiate from stem callus after 12-14 days. The maximum number of shoot buds were produced in the MS medium supplemented with 5 μ L IAA and 50 μ L BAP. Whereas minimum number of shoot buds were produced in the MS medium supplemented with 20 μ L IAA and 60 μ L BAP because the high concentration of growth hormones retard shoots formation and initiate rooting. The response of shoot initiation was good from stem callus.

Response of leaf callus to shoot induction

2 weeks old callus of leaf explants was cultured on the shoot induction medium (SIM) supplemented with different concentrations of auxins and cytokinins.

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 10 μ L IAA+55 μ L BAP/500ml MS media.
- 3) 20 μ L IAA+60 μ L BAP/500ml MS media.

Shoot buds were produced in the MS medium supplemented with 5 μ L IAA and 50 μ L BAP. Whereas minimum number of shoot buds were produced in the MS medium supplemented with 20 μ L IAA and 60 μ L BAP because the high concentration of growth hormones retard shoots formation and initiate rooting. The response of shoot initiation was excellent from leaf callus.

Response of root explants to shoot induction

2 weeks old callus of root explants was cultured on the shoot induction medium (SIM) supplemented with different concentrations of auxins and cytokinins.

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 10 μ L IAA+55 μ L BAP/500ml MS media.
- 3) 20 μ L IAA+60 μ L BAP/500ml MS media.

Shoot buds started to initiate from root callus after 12-14 days. The maximum number of shoot buds were produced in the MS medium supplemented with 5 μ L IAA and 50 μ L BAP. Whereas minimum number of shoot buds were

produced in the MS medium supplemented with 20 μ L IAA and 60 μ L BAP because the high concentration of growth hormones retard shoots formation and initiate rooting. The response of shoot initiation was poor from root callus.

Response of cotyledon explants to shoot induction

2 weeks old callus of cotyledon explants was cultured on the shoot induction medium (SIM) supplemented with different concentrations of auxins and cytokinins.

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 10 μ L IAA+55 μ L BAP/500ml MS media.
- 3) 20 μ L IAA+60 μ L BAP/500ml MS media.

Shoot buds started to initiate from cotyledon callus after 12-14 days. The maximum number of shoot buds were produced in the MS medium supplemented with 5 μ L IAA and 50 μ L BAP. Whereas minimum number of shoot buds were produced in the MS medium supplemented with 20 μ L IAA and 60 μ L BAP because the high concentration of growth hormones retard shoots formation and initiate rooting. The response of shoot initiation was very good from cotyledon callus.

Root Induction

Root Induction from stem explants

Shoot buds derived from callus of stem explants were cultured on root induction medium (RIM) having different concentrations of auxins and cytokinins.

- 1) 10 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 20 μ L IAA+55 μ L BAP/500ml MS media.
- 3) 30 μ L IAA+60 μ L BAP/500ml MS media.

Root formation was started from stem callus after 2-3 weeks. The highest number of roots were produced in the MS medium supplemented with 10 μ L IAA and 50 μ L BAP. Whereas lowest number of roots were produced in the MS medium supplemented with 30 μ L IAA and 60 μ L BAP because the high concentration of growth hormones retard roots formation. The response of root formation was good from stem callus.

Root Induction from leaf explants

Shoot buds derived from callus of leaf explants were cultured on root induction medium (RIM) having different concentrations of auxins and cytokinins.

- 1) 10 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 20 μ L IAA+55 μ L BAP/500ml MS media.

3) 30 μ L IAA+60 μ L BAP/500ml MS media.

Root formation was started from leaf callus after 2-3 weeks. The highest number of roots were produced in the MS medium supplemented with 10 μ L IAA and 50 μ L BAP. Whereas lowest number of roots were produced in the MS medium supplemented with 30 μ L IAA and 60 μ L BAP because the high concentration of growth hormones retard roots formation. The response of root formation was excellent from leaf callus.

Root Induction from root explants

Shoot buds derived from callus of root explants were cultured on root induction medium (RIM) having different concentrations of auxins and cytokinins.

- 1) 10 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 20 μ L IAA+55 μ L BAP/500ml MS media.
- 3) 30 μ L IAA+60 μ L BAP/500ml MS media.

Root formation was started from root callus after 2-3 weeks. The highest number of roots were produced in the MS medium supplemented with 10 μ L IAA and 50 μ L BAP. Whereas lowest number of roots were produced in the MS medium supplemented with 30 μ L IAA and 60 μ L BAP because the high concentration of growth hormones retard roots formation. The response of root formation was not so good from root callus.

Root Induction from cotyledon explants

Shoot buds derived from callus of cotyledon explants were cultured on root induction medium (RIM) having different concentrations of auxins and cytokinins.

- 1) 10 μ L IAA+50 μ L BAP/500ml MS media.
- 2) 20 μ L IAA+55 μ L BAP/500ml MS media.
- 3) 30 μ L IAA+60 μ L BAP/500ml MS media.

Root formation was started from cotyledon callus after 2-3 weeks. The highest number of roots were produced in the MS medium supplemented with 10 μ L IAA and 50 μ L BAP. Whereas lowest number of roots were produced in the MS medium supplemented with 30 μ L IAA and 60 μ L BAP because the high concentration of growth hormones retard roots formation. The response of root formation was very good from cotyledon callus.

Hardening

Hardening of regenerated plants was the last and most critical step of plant tissue culture. Completely grown plants with 90% survival rate were removed from jars and their roots were washed with distilled water. To get rid from fungal

infection roots were dipped in a fungicide solution. Regenerated plants were transferred to pots containing compost for more shoot elongation and healthy root development. Compost contains plant litter, animal manure, sand and soil. It provides large amount of nutrients required by the plantlets. Initially pots were covered with polyethylene bags for one week, and were kept in the growth room at 28 \pm 2 $^{\circ}$ C. After one week a hole was created in the plastic bags for aeration and to make the plantlets resistant from environmental stress.

DISCUSSION

Chili is an important vegetable and spice crop of the world, commonly known as pepper, sweet pepper or bell pepper. Chili has been widely used for the preparation of all kinds of cuisines throughout the world. It belongs to family *Solanacea*, frequently known as night shades. Potato, tobacco and tomato are also the members of this family. This plant is originated from Central and South America as a wild crop around 7500BC (Grubben and EI Tahir 2004). The plants of the Capsicum genus are represented by the terms; chili, chile, chili, paprika, aji, pepper and capsicum (DeWitt and Bosland, 1993). Capsaicinoids which are located in the placenta of the fruit are responsible for determining pungency of chili peppers (Bora *et al.*, 2014). Green chilies contain seven times more vitamin C as compared to oranges. On the other hand, red chilies contain more quantity of Vitamin A than that of carrots (Marin, Ferreres *et al.*, 2004). A wide range of bioactive compounds are present in chilies which display anti-oxidant, anti-microbial, anti-inflammatory and anti-cancerous properties (Khan *et al.*, 2014). Although Capsicum is important economically but chili pepper is sensitive to regeneration system particularly at the shoot elongation phase in contrast to other solanaceous species including potato, tobacco and tomato which show great ability for regeneration. Additionally chili peppers are extremely vulnerable to viral and fungal pathogens which cause significant damage to these crops. Low germination rate of seeds, short viability time period and threat of various pathogenic attacks are also some major disadvantages of chilies. As a consequence, *in vitro* regeneration methods are used which gave methods of asexual multiplication.

In vitro culture techniques are simple, useful and profitable due to which large number of disease free and genetically pure plants can be achieved within a short time for better quantity and quality of crops. Chili plants lack natural vegetative propagation due to which genetic purity is lost and *in vitro* culture techniques provide solution for it (Ashrafuzzaman *et al.*, 2009). The present study was scheduled to produce reasonable, efficient and high yielding protocols for regeneration by the use of various explants of *Capsicum annuum* L. Chili plants are regenerated from different explants like cotyledons, hypocotyls, zygotic embryos, mature seeds, shoot tips, leaves, embryonal leaves, stems and roots (Agrawal, 1983). In our research chili plants are regenerated by using stem, leaves, roots and cotyledons as source of explant. To determine the time duration of callus initiation one variety of chili SVHA 1182 was used. For this purpose three types of MS basal media were prepared having different concentrations of plant hormones (PGRs).

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media
- 2) 5 μ L NAA+50 μ L BAP/500ml MS media
- 3) 5 μ L IAA+50 μ L 2,4-D/500ml MS media

After 10 days callus initiated was observed from all the three explants (stem, leaf, root and cotyledon). The rate of callus induction was maximum on media supplemented with high concentration of BAP (50 μ L) and low concentration of IAA (5 μ L). After the development of compact and hard texture callus, it was cut into small pieces. When these pieces were transferred onto fresh medium friability of callus was increased. Shoot regeneration was observed by using stem, leaf, root and cotyledon as source of explant. 2 weeks old callus of stem, leaf, root and cotyledon explants were cultured on the shoot induction medium (SIM) supplemented with different concentrations of auxins and cytokinins.

- 1) 5 μ L IAA+50 μ L BAP/500ml MS media
- 2) 10 μ L IAA+55 μ L BAP/500ml MS media
- 3) 20 μ L IAA+60 μ L BAP/500ml MS media

Shoot buds started to initiate from callus after 12-14 days. Maximum shooting was observed on MS medium supplemented with 5 μ L IAA and 50 μ L BAP. Whereas minimum number of shoot buds were produced in the MS medium supplemented with 20 μ L IAA and 60 μ L BAP. *In vitro* regenerated shoots were used for root induction by using three types of root induction

medium (RIM) having different concentrations of auxins and cytokinins.

- 1) 10 μ L IAA+50 μ L BAP/500ml MS media
- 2) 20 μ L IAA+55 μ L BAP/500ml MS media
- 3) 30 μ L IAA+60 μ L BAP/500ml MS media

Root induction was initiated from callus of stem, leaf, root and cotyledon after 2-3 weeks. The highest number of roots were produced in the MS medium supplemented with 10 μ L IAA and 50 μ L BAP. Whereas lowest number of roots were produced in the MS medium supplemented with 30 μ L IAA and 60 μ L BAP. The most and most sensitive step of plant tissue culture was hardening which requires great care. Rooted plantlets were removed from jars and were transferred to pots containing compost for more shoot elongation and healthy root development. Initially pots were covered with polyethylene bags for one week, and were kept in the growth room at 28 \pm 2 $^{\circ}$ C. After one week a hole was created in the plastic bags to make the plantlets resistant from environmental stress.

CONCLUSION

Capsicum annuum L contains a little, annual shrub whose stem grows up to a meter in height, naturally found in Central American region and is used as a main pungency ingredient in the cuisine of Mexican for decades. This plant belongs to tropical and sub-tropical region. Chilies have gained a great value in food industry due to its tremendous nutritive value. Chilies have an important role in the pharmaceutical industry because of the presence of bioactive compounds and vital nutrients. Capsaicinoids which are located in the placenta of the fruit are responsible for determining pungency of chili peppers. Conventional breeding methods have helped a lot in increasing chili crop production but some disadvantages are also present in this method such as low germination rate and short viability time period. Therefore tissue culture techniques are utilized for the production of disease free plants with desired characteristics. Different parts of *Capsicum annuum* L. are used as explants for the development of an efficient regeneration system. For the growth of chili seeds MS media was used. Sterilization of these explants was done by using 10% bleach for about 5 minutes followed by

rinsed with autoclaved distilled water three times for complete removal of bleach from seeds for the dryness of seeds. Then the seeds were placed on an autoclaved filter paper. After that seeds were placed on MS media plates and covered with cling film to avoid contamination. Plates were placed in the culture room at 26-28°C temperature under a 16/8h (light/dark) regime. Seeds started to germinate after 7 days. After 2 weeks healthy seedlings were selected for callus induction. For that purpose callus induction media supplemented with various plant growth regulators was prepared. Callus started to initiate from the sides of the explants (stem, leaf, root and cotyledon) after 10 days. Then the callus was sub-cultured on next freshly prepared media having same concentration of growth hormones for its maintenance and multiplication. Extensive sub-culturing was avoided because when callus got older it only increased in mass but no shooting induction was observed. Completely grown plantlets were transferred to small pots filled with compost and covered with the help of polythene bag to protect the plantlets from environmental stress and kept in the growth room at 26 + 2°C. Plantlets were washed with distilled water after 4-5 days. Tissue culture technique was found to be efficient and quick with the help of which healthy and disease free chili plants can be developed. In conclusion, tissue culture techniques could be useful for the development of transgenic *Capsicum annum*. This technique also helps in producing best quality fruits and resistant chili plants that fight against many bacterial, fungal and viral diseases.

REFERENCES

- DeWitt, D., Bosland, P. W. The pepper garden. Berkeley, CA: Ten Speed Press., 1993: **240**.
- Grubben, G. and I. M. El Tahir. *Capsicum annum* L. *PROTA*, 2004; **2**: 154-163.
- Kothari, S.L., A. Joshi, S. Kachhwaha and N. Ochoa-Alejo. Chilli peppers – A review on tissue culture and transgenesis. *Biotechnol. Advances.*, 2010; **28**: 35-48.
- Basu, S. K. and A. Krishna De, *Capsicum*: historical and botanical perspectives. *Capsicum: the genus Capsicum*, 2003; **33**: 1-15.
- Valadez-Bustos, M. G., G. A. Aguado-Santacruz, G. Carrillo-Castañeda and A. R. Paz, In vitro propagation and agronomic performance of regenerated chili pepper (*Capsicum* spp.) plants from commercially important genotypes. *In Vitro Cell. Dev. Biol-Plant*. 2009; **45**: 650-658.
- Khan, M. A., M. A. Asghar, J. Iqbal, A. Ahmed and Z. A. Shamsuddin, Aflatoxins contamination and preservation in red chillies (*Capsicum annum* L.) in Pakistan. *Food Add. Cont.*, 2014; **7**: 1-6.
- Kosuge, S. and M. Furuta. Studies on the pungent principle of *Capsicum*. Part XIV. Chemical constitution of the pungent principle. *Agr. Biol. Chem.*, 1970; **34**: 248-256.
- Monnerville, L.A. Determination of capsaicin and dihydrocapsaicin by micellar electrokinetic capillary chromatography and its application to various species of *Capsicum*. *J. Chromatogr. A.*, 1999; **836**: 293-302.
- Steward, F.C., M. Mares and J.S. Mears. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *Am. J. Bot.*, 1958; **45**: 705-8.
- Shibli, R. A., Kushad, M., Yousef, G. G., & Lila, M. A. Physiological and biochemical responses of tomato microshoots to induced salinity stress with associated ethylene accumulation. *Plant growth regulation*, 2007; **51**(2), 159-169.
- Grattan, D. R. Behavioural significance of prolactin signalling in the central nervous system during pregnancy and lactation. *Reproduction*, 2002; **123**(4), 497-506.
- Baire, B., & Kane. Catalyst free, three-component approach for unsymmetrical triarylmethanes (TRAMs). *Tetrahedron Letters*, 2011; **57**(48), 5381-5384.
- Ashrafuzzaman, M., M. M. Hossain., M. S. Haque., S. M. Shahidullah. And Shahin-uz-zaman. Regeneration potential of seedlings explants of chilli (*Capsicum annum*). *J. A. Biotechnol.*, 2009; **8**: 591-596.
- Bhagowati, R. R. and S. Changkija. Genetic variability and traditional practices in Naga King landraces of Nagaland. *J. Asian Agri-History*. 2009; **13**: 171-1803.
- Evans, D., W. R. Sharp, P. V. A. Macmillan, Handbook of plant cell culture. V. 1: Techniques for propagation and breeding. *J. Agric. Eng. Res.*, 1983; **12**(2): 67-78.
- Agrawal, M., Samala, R., Doddamani, R., Agrawal, D., & Chandra, S. P. The role of selective dorsal rhizotomy in the management of post-traumatic spasticity: systematic review. *Neurosurgical Review*, 1983; 1-9.