Research Article

Response of Different (*Capsicum annuum* L) Genotypes for Callus Induction, Plant Regeneration and Plant Transformation

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Abstract | Chilli (*Capsicum annuum*), which belongs to the family *Solanaceae*, is an important spice crop cultivated in tropical and subtropical countries. A Protocol for chili plant regeneration with hypocotyl and cotyledon explants was established. The study was conducted to observe the effect of genotypes, culture conditions and growth regulators on plant regeneration of chili pepper genotypes (Seedex Pepper (SP), Loungi, Tatapuri, and Sanam) grown in Pakistan including. For both hypocotyl and cotyledon explants, SP was found to be the most sensitive among the tested genotypes tested. Maximum mean callus induction rate of hypocotyl was 76.6% in SP followed by TP (73.3%); Sanam (57.3%); Loungi (43.3%) respectively. Whereas in case of cotyledon maximum inductions rate was found in Loungi (62.0%) followed by SP (60); Sanam (57.3); TP (46) individually. According to the observations maximum plant regeneration was found in genotype SP (9%), followed by Loungi (1.8%); Sanam (1.5%) but no plant regeneration was found in TP. *A. tumefaciens* LBA4404 with the 35S GFP/pFGC construct were used to evaluate hypocotyl and cotyledon explants for transformation. The effects of co-cultivation at different temperatures (22 and 25°C), photoperiods (16 hours of light, 8 hours of darkness, and full darkness), and co-cultivation periods were studied. GFP assays revealed that the putative transgenic calli were not transformed and died after 40-60 days. The experiment was repeated ten times to see the success rate of the regeneration system. In this study a protocol for chili plant regeneration was developed for callus induction, plant regeneration, and plant transformation. Because developing a viable chilli tissue culture and plant regeneration system is difficult, this study was designed to help develop one. SP was found to be the most suitable among the four genotypes used for regeneration.

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Introduction

Chili peppers are members of the Capsicum genus, which belongs to the family *Solanaceae*, *ae* (Kothari *et al*., 2010; Jeong *et al*., 2011). There are 5 cultivated and 26 wild Capsicum species in the genus Capsicum (Wahyuni *et al*., 2013). Chili is an important cash crop in Pakistan, with a total cultivated area

of 63.6000 hectares, 52.8000 hectares in Sindh, and an annual production of 171.8000 tonnes (Fayyaz *et al*., 2019). Chili accounts for 19% of the total vegetable cultivation area and is cultivated on 38.4 thousand hectares with a yield of 90.4 thousand tonnes (Shah *et al*., 2008). Different varieties of sweet peppers and pungent chili peppers are cultivated and widely used around the world (Ur-Rahman, 2018) as dried powders of various colors, but they suffer significant losses due to virus infection (Amelia *et al*., 2020). 45 diverse virus species have been identified to infect chilies/peppers worldwide (Shah *et al*., 2008).

Genetic engineering holds great promise for effective plant virus control (Yadav *et al*., 2009). Since the pepper plant has so many useful characteristics, it was deemed appropriate to genetically modify it. Genetic transformation is now a popular method for injecting genes into a wide range of plant species, including vegetable and fiber crops (Ban *et al*., 2009). Current progress in plant genetics and biotechnology is highly dependent on the use of in-vitro tissue culture, requiring the development of an efficient plant regeneration system (Yancheva and Kondakova, 2018). Somatic embryogenesis (SE) by callus induction is particularly valuable among the various systems used (Gaj, 2004). It also offers opportunities for *In vitro* production of plants through clonal propagation and genetic modification through genetic transformation. As a result, there is a growing number of protocols explaining successful in-vitro regeneration that are being published (Kothari *et al*., 2010; Li *et al*., 2003; Gururaj *et al*., 2004; Ashrafuzzaman *et al*., 2009).

Several protocols for inducing in-vitro plant regeneration in chili pepper are available (Kothari *et al*., 2010; Li *et al*., 2003; Mihálka, 2006; Lee *et al*., 2004; Hasnat *et al*., 2008). However, some of these studies indicate that genotype (Phillips and Garda, 2019) culture medium composition, explant source, and environment all have a strong influence on the regeneration process.

Among them, the genotype and nutrient composition (Lee *et al*., 2006) are regarded to be the major sources of variation in *in-vitro* culture (Li *et al*., 2003) and had been studied. Pepper is a member of the Solanaceae family, which has members that are sensitive to tissue culture and transformation procedures; however, pepper is known as a tremendously problematic and resistant species for in-vitro regeneration and genetic transformation (Kothari *et al*., 2010).

The gene transfer in chili is difficult and there are several obstacles i) whole plant regeneration through tissue culture ii) procurement of transformed tissues iii) plant regeneration after transformations (Kothari *et al*., 2010). In the last ten years, scientists have made significant progress in the transformation of chilies all over the world (Kothari *et al*., 2010; Hasnat *et al*., 2008; Mihálka, 2006). Transgenic pepper expressing the coat protein gene of CMV (Morroni *et al*., 2008) and plants that expressed CMV satellite RNA (Li *et al*., 2003) were obtained with low regeneration and transformation efficiencies. However, the published protocols could not be repeated in other laboratories. This study was therefore conducted to screen commercial genotypes of local (Pakistani) chili for callus response and the development of an efficient in-vitro clonal propagation protocol. This protocol demonstrates the genotype independent response for morphogenic callus formation and genotype-dependent response for plant regeneration. The effects of various factors on genetic transformation in a local chili pepper genotype were also studied. So therefore, this study was aimed to develop a successful callus induction, plant regeneration, and plant transformation system for chili pepper grown in Pakistan.

Materials and Methods

Plant material, Seed Germination, and Explant preparation

The primary goal of this study was to investigate the effects of various plant growth regulators, genotypes, and explants on chili tissue culture, as no reports of such effects on chili regeneration had previously been published in Pakistan. Commercial genotypes of *C. annuum* (Loungi and Sanam) seeds were taken from Ayyub Agricultural Research Institute, Faisalabad Pakistan and *C. annuum* genotypes [Seedex pepper (SP) and Tatapuri (TP)] seeds from Sindh Horticulture Research Institute, Mirpurkhas were obtained and used in this study. Surface sterilized chili seeds were planted in Murashige and Skoog medium (MS-zero) medium. After 12 days of germination, the hypocotyls and cotyledons of seedlings were removed and used as explants for callus induction. The hypocotyl explants were cut into 3.5-4.5 mm sections, and the cotyledons were cut into two parts transversely. To keep the cut edges of the explants from drying out, they were cultured immediately.

Table 1: *Chili callus induction medium (ChC).*

Chili Callus induction media	Basal media Φ	Sucrose (g/L)	BA (mg/L)	IAA (mg/L)	NAA (mg/L)	Agar (mg/L)
ChC1	MS salts + vitamins	30	2.0	-	1.0	9.0
ChC2	MS salts + vitamins	30	3.0	0.5	-	8.5
ChC3	MS salts + vitamins	30	4.0	0.5	-	9.0

Φ MS salts and vitamins (Phyto Technology USA, Prod NO: M404)

Table 2: *Chili shoot regeneration medium (ChSR).*

Chili Shoot Regeneration media	Basal medium Φ	Sucrose (g/l)	BA (mg/l)	NAA (mg/l)	GA3 (mg/l)	Agar (mg/l)
ChSR1	MS salts + vitamins	30	5.0	0.05	-	9.0
ChSR2	MS salts + vitamins	30	4.0	0.05	2	8.5
ChSR3	MS salts + vitamins	30	3.0	0.05	-	9.0

Φ MS salts and vitamins (Phyto Technology USA, Prod No: M404)

Agrobacterium-mediated genetic transformation in chili pepper (C. annuum L.)

Hypocotyl and cotyledon explants of *C. annuum* SP were obtained and a single clone of *A. tumefaciens* LBA 4404 35S GFP/pFGC (Provided by Molecular Virology and Gene silencing Lab, NIBGE) was inoculated in 20 mL LB liquid medium supplemented with 50 mg/L kanamycin, 100 mg/L streptomycin and cultured on a rotary shaker at 28°C, 180 rpm, for 24 h. The bacterial cells were then centrifuged and the pellet suspended in MS0 liquid medium (MS medium without agar) to O.D.600=0.38–0.42. Hypocotyl and cotyledon explants were inoculated with the cultures of *A. tumefaciens* LBA4404 having 35S GFP/pFGC construct for 8–10 min, followed by co-culture on ChC2 medium at different temperatures (22 and 25°C), photoperiod (16h light 8h dark, and complete darkness) and co-cultivation periods. Explants were separated from co-culture medium and kept on ChC2 selection medium [ChC2 medium, glufosinate-ammonium (Basta, 4 mg/l) and cefotaxime (50 mg/l)] and incubated. Explants were subcultures after every 2-3 weeks and placed on ChC2 selection medium. GFP fluorescence was observed in putative transgenic calli using a 100-W long-wave UV lamp (Blak-Ray Model B 100YP; UV Products). The total genomic DNA of the callus of chilies was extracted by the CTAB method. The transgene in callus was confirmed through PCR with specific primers of the transgene. Calli became brownish and dead after 40 days. 200 hypocotyl and 150 cotyledon explants of both genotypes in 10 different batches (with different experimental conditions) were evaluated in these experiments.

Culture Medium and condition

At a temperature of 25± 2°C and a photoperiod of 16/8 hours, hypocotyl and cotyledon explants were put on three separate formulations of chili callus induction media (ChC1, ChC2 and ChC3) (Table 1). Calli from each mixture was divided into three parts and cultured on three separate formulations of (ChSR1, ChSR2, and ChSR3) shoot regeneration media for plant regeneration (Table 2). Tissue culture grade agar (0.8 percent w/v) was used to solidify all of the media and pH was adjusted to 5.8 before autoclaving. The disinfected medium was poured onto Petri dishes, sealed with parafilm. Individually excised elongated multiple shoots (3–4 cm long) were cultured on an MS-zero chili root propagation medium for fifty days and allowed to grow roots. The rooting agar was washed away from the plantlets, and the plantlets were transferred to pots containing a soil: Vermiculite (1:1) mixture. Fifty hypocotyl explants and fifty cotyledon explants (10 explants in 5 replicates) of each genotype were used in these experiments. The mean ± SE values have been calculated from all the data of experiments using Statistix 8.1.

Results and Discussion

Callus induction

The data for the callus induction was recorded after 30-32 days. Effect of four chili genotype on callus induction after 32 days showed that callus of SP genotype grow fast as compared to other three genotypes followed byTP, Sanam, and Loungi (Figure 1). Callus was induced from both hypocotyl and cotyledon explants from all chili genotypes. Factorial analysis of variance (ANOVA) test was carried out to detect differences among the factors tested. The data were ana-

lyzed and statistical analysis revealed that there was no significant difference in the type of explants and medium used in this study on the frequency of callus induction (Table 3). For all four genotypes, callus induction from cotyledons was quicker than hypocotyls. Calli appeared from the cut edges of both hypocotyls and cotyledon explants after about 3-4 weeks of culture with variable proliferation rates. Maximum mean callus induction rate if hypocotyl was with SP (76.6%) followed by TP (73.3%); Sanam (57.3%); Loungi (43.3%) respectively. whereas in case of cotyledon maximum inductions rate was found in Loungi (62.0%) followed by SP (60); Sanam (57.3); TP (46) individually. graphical representation of Interaction of genotype and explants (hypocotyl and cotyledon) response on chili callus induction after 32 days is shown in (Figure 2). Most of the calli obtained were compact with an opaque or yellowish color and some were soft, watery, morphogenic calli with translucent or light yellow. Callus was formed in both explants tissues (hypocotyl and cotyledon) segments of 2 spinach cultivars, but the percentage of callus formation was not variable in different explants.

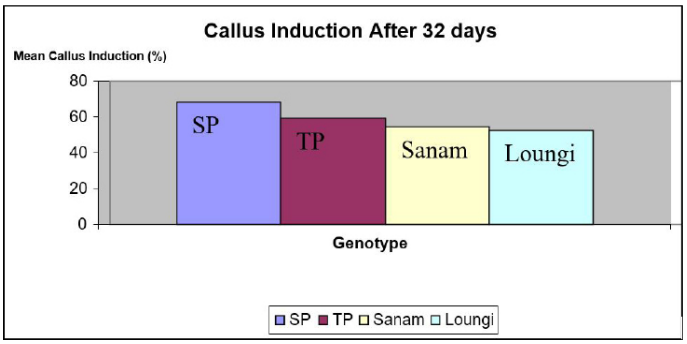

Figure 1: *Effect of four genotype (SP, TP, Sanam, and Loungi) on chili callus induction.*

Table 3: *Interaction of genotype and explants (hypocotyl and cotyledon) response on chili callus induction.*

Explants	Genotype	Mean (%)	Homogeneous Groups
Hypocotyl	SP	76.6	A
Hypocotyl	TP	73.3	Ab
Cotyledon	Loungi	62.0	Bc
Cotyledon	SP	60.0	C
Cotyledon	Sanam	57.3	CD
Hypocotyl	Sanam	51.3	CDE
Cotyledon	TP	46.0	DE
Hypocotyl	Loungi	43.3	E

A combination of genotype, explants and callus induction medium response on chili callus induction is shown in (Figure 3). Loungi showed the lowest callus induction potential (52.6%), while SP showed maximum callus induction growth. Among the media composition, callus induction medium having (IAA 1 mg/L + BA 3 mg/L) was found to be most effective for callus induction but the differences were statistically non-significant. Callus obtained from this medium was quite good in texture and friable in nature than another medium, SP hypocotyl explants showed maximum callus induction on medium containing (IAA 1 mg/L + BA 3 mg/L) while Sanam cotyledons showed maximum callus induction on a medium having (IAA 1 mg/L and BA 2 mg/L). Rhizogenic calli were obtained from hypocotyl explants of Loungi that were non-regenerable in ChC1 medium.

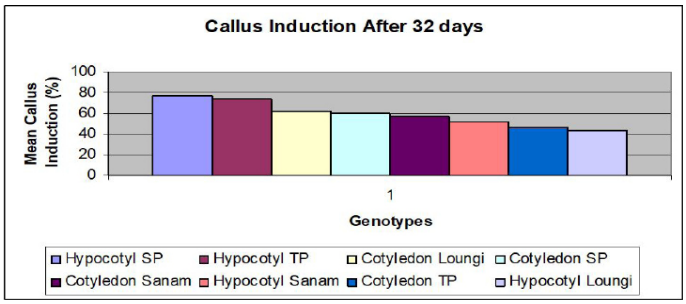

Figure 2: *Interaction of genotype and explants (hypocotyl and cotyledon) response on chili callus induction.*

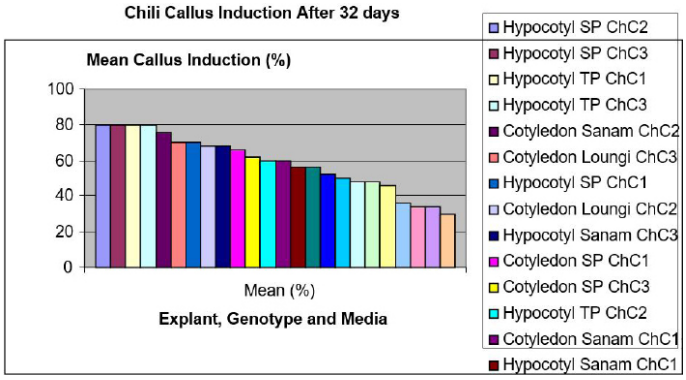

Figure 3: *Combination of genotype, explants and callus induction medium response on chili callus induction.*

Chili Plant regeneration

Three different chili shoot regeneration media (ChSR1, ChSR2 and ChSR3) based on MS salts supplemented with 1% (w/v) sucrose, 0.05 mg/lNAA, 3-5 mg/l BA alone or in the combination of GA3 2 mg/L were used to test the suitable medium for chili plant regeneration from calli. A significant difference at (P >0.05) in the plant regeneration system was observed in the genotypes. All 4 genotypes showed different regeneration responses on three different combinations but none of them gave any regeneration response on MS without any hormones. SP showed the highest regeneration efficiency and TP did not re-

spond to plant regeneration. Effect of four genotype (SP, TP, Sanam, and Loungi) on chili plant regeneration is shown in (Figure 4). After 32 days of callus induction plant regeneration of each variety was observed and recorded. According to the observations maximum plant regeneration was found in genotype SP (9%), followed by Loungi (1.8%); Sanam (1.5%) respectively. As compared to other three genotypes fourth one (TP) showed no plant regeneration at all.

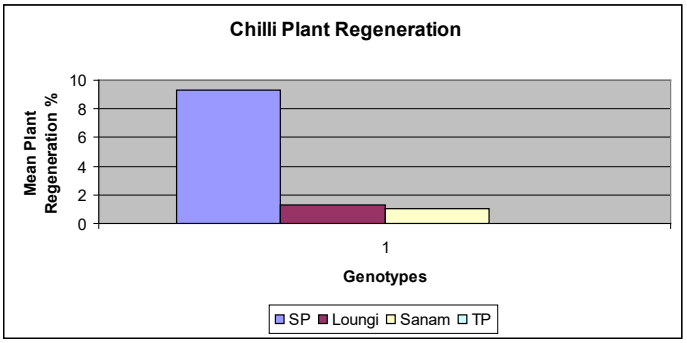

Figure 4: *Effect of four genotype (SP, TP, Sanam, and Loungi) on chili plant regeneration.*

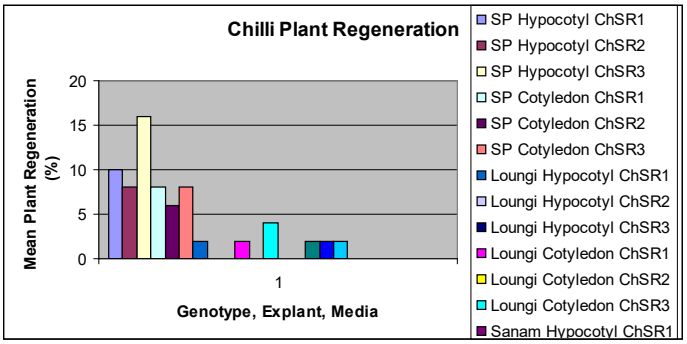

Figure 5: *Effect of combination of genotype, explant and shoot regeneration medium on plant regeneration from chili calli.*

Interestingly the genotype SP which showed a high regeneration potential also performed better in callus induction. Effect of combination of genotype, explant and shoot regeneration medium on plant regeneration from chili calli as shown in (Figure 5). Shoot regeneration was started 30-40 days after culturing on the shoot regeneration medium. Regeneration frequency varied between 0 and 16 % in hypocotyl explants. The highest regeneration frequency (16 %) was obtained from genotype SP. In cotyledon explants, maximum regeneration frequency (8.0 %) was obtained in genotype SP.

Effect of different factors on Agrobacterium-mediated plant transformation in chili pepper (C. annuum L)

One of our research goals was to develop a transformation system that allows homogeneous transgene expression of local chili genotype with GFP/pFGC. The hypocotyl and cotyledon explants (SP genotypes) from 10-15 days old seedlings were placed on ChC2 medium supplemented with different concentrations (0, 2, 3, 4, 5, and 6 mg/L) of glufosinate-ammonium (Basta). After 25 days the callus differentiation rates of the explants were observed. The explants tested induced callus on Basta-free medium. But only the cotyledon explants could tolerate a basta concentration of (4 mg/L). When the level of basta was (5 mg/L) or higher, calli could not be induced for any explants. Hence, 5 mg/L Basta considered being the minimal lethal dose.

In the preliminary experiments of this study, various factors that influence the efficiency of T-DNA delivery in the Chili plant were assessed. Factors include two explant types *A. tumefaciens* cells inoculation at 22 and 25°C, as well as photoperiod (16h light/ 8h dark and complete darkness) and periods of co-cultivations. Explants were inoculated and co-cultured with *A. tumefaciens* LBA4404 having 35S GFP/ pFGC construct for 8–10 minutes. Transient GFP expression was observed with very low frequencies in hypocotyl explants after 2 or 3 days of co-culture under complete darkness. Transient GFP gene expression was lost completely from the tissue 20 days after transformation. Calli became brownish and dead after 40-45 days (Figure 6). PCR analysis confirmed that the transgene was present in the putative transgenic callus (Figure 7).

The concept of *In vitro* culture which exploits the ability of plant cells to regenerate was proposed by (Haberlandt, 2003) and demonstrated for the first time by (Steward and Mear,1958). Previous studies have revealed the several aspects of inherent problems that are associated with in vitro studies of chili *i.e,* non-availability of morphogenic calli, severe recalcitrance, less defined shoot buds, a genotypic requirement that can expose to tissue culture and plant improvement through genetic transformation system (Kothari *et al.*, 2010; Rajam *et al.*, 2021). Chili has less ability to regenerate while other *Solanaceae* crops such as tomato, tobacco, potato are frequently used as model systems due to their ability to regenerate plants. Regardless of the fact many findings had been conducted for the relative success of the regeneration system in different crops (Kim *et al.*, 2008; Kumar *et al.*, 2017; Keshavareddy *et al.*, 2018) on shoot morphogenesis in chili but genetic engineering is still restricted by the low morphogenetic potential of

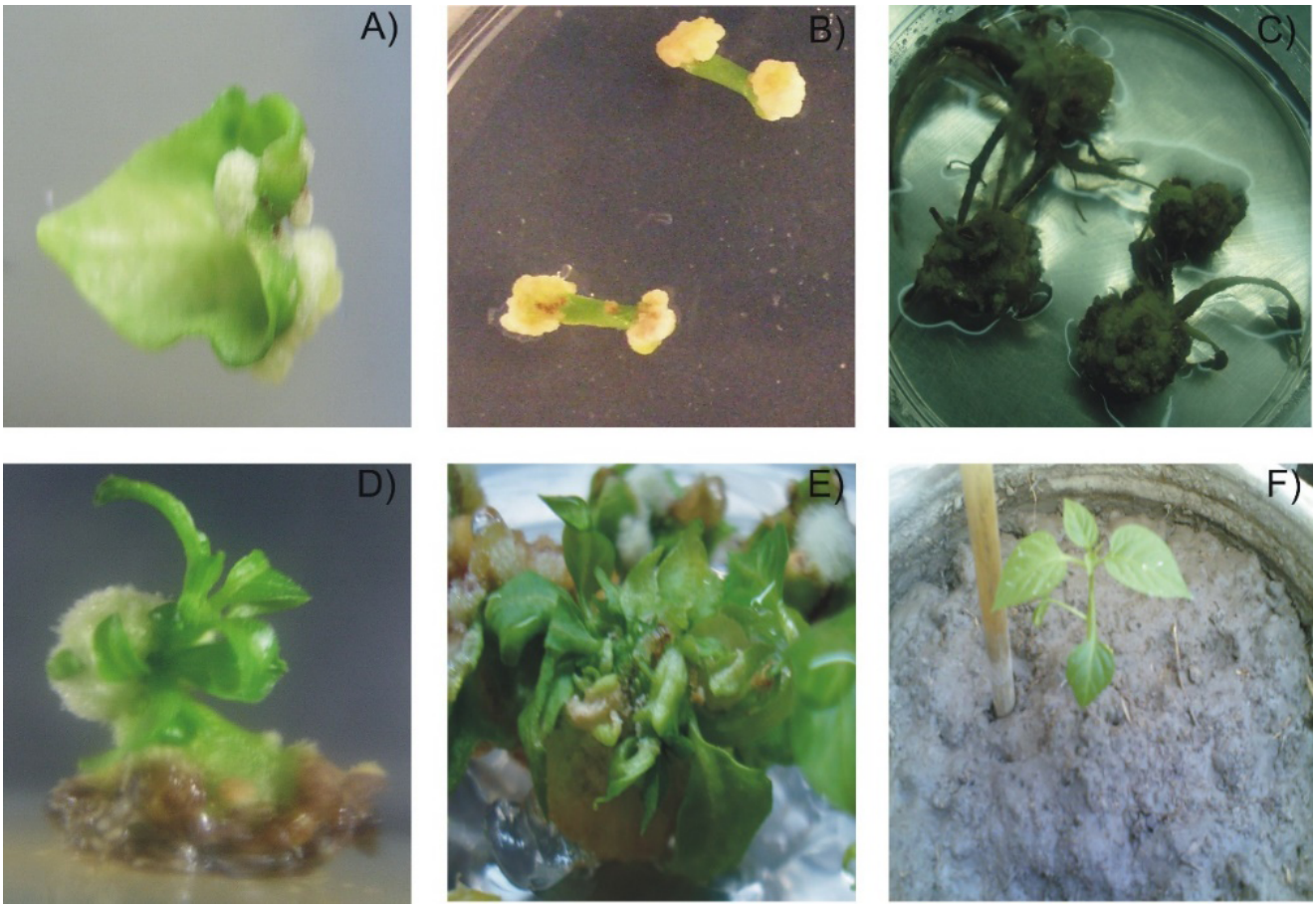

Figure 6: *Different step in chili tissue culture,* **A)** *Callus induction from cotyledon explants,* **B)** *Callus induction from hypocotyl explants,* **C)** *Rhizogenic callus,* **D)** *Shoot regeneration,* **E)** *Multiple shoot induction,* **F)** *regenerated plant.*

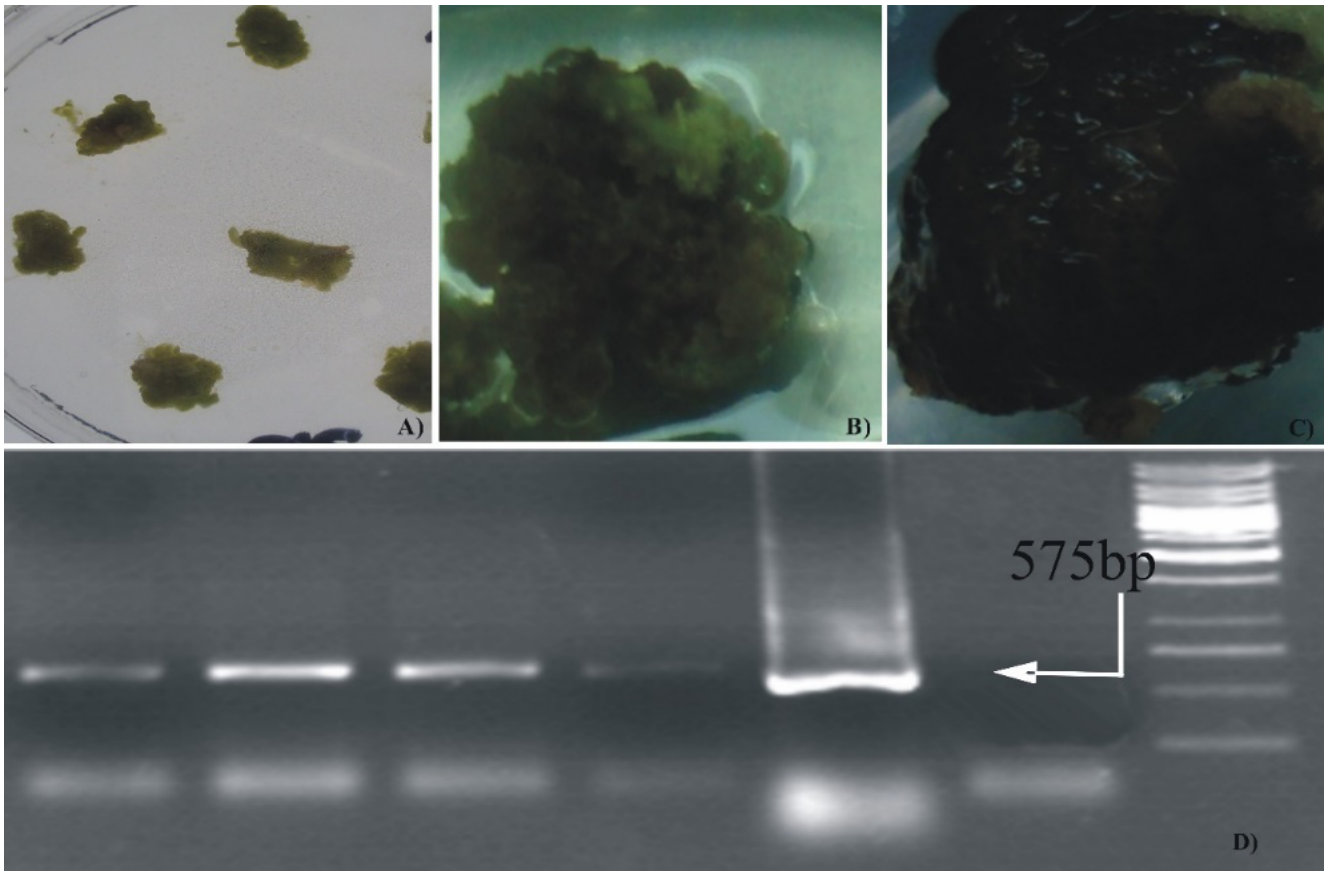

Figure 7: *Plant transformation in chilies and transgene analysis.* **A, B** *and* **C)** *Chili callus after inoculation with A. tumefaciens LBA4404 35S GFP/pFGC,* **D)** *PCR confirmation of the transgene using specific primers of the transgene in calli.*

these species (Mihálka, 2006; Yunita et al., 2021). Explant selection at a given stage of the plant, as well as changes in nutritional media and additives, could all aid to reduce recalcitrance (Kothari et al., 2010; Bridgen et al., 2018). The effort has been made to identify suitable explants in chili for morphogenic callus induction and subsequent plant growth. Different types of explants comprise of cotyledons, hypocotyls, leaves, shoot tips, and roots, *etc.* have been employed for plant regeneration in solanaceous family (Nowaczyk and Kisiala, 2006; Peddaboina and Thamidala, 2006; Kumar and Kaur, 2017).

Callus induction

In this study hypocotyl and cotyledon explants were used from 10-15 days old seedlings. No significant difference was observed for the type of explants for callus formation and plant regeneration. But in this study by optimizing a suitable medium composition, morphogenic callus induction was achieved from hypocotyl explants of SP chili genotype. The results of this study were supported by the finding of Valadez-Bustos *et al.* (2009) and Ashrafuzzaman *et al.* (2009) which also showed that callus induction and shoot initiation was higher in hypocotyls and embryos than cotyledons. Auxins and cytokinins are mandatory to induce cell division and growth in the tissue culture system (Kothari *et al.*, 2010).

Screening of MS and B5 media for Callus induction

In this study, four different chili genotypes were tested for morphogenic callus induction on modified MS and B5 media containing different concentrations of BA (2-4 mg/L) in combination with IAA (0.5 mg/L) or BA 2.0mg/L in combination with NAA (1.0 mg/L). Callus induction from hypocotyl explants ranged from 70-80 % for SP; 30-60 % for Sanam; 60-80 % for TP and 34-70 % for Loungi on three mediums. Callus induction from cotyledon explant ranged from 52-66 % for SP; 36-76 % for Sanam; 34-56 % for TP and 48-70 % for Loungi on three different media. It can be concluded from these results that increasing BA concentration in the callus induction media generally has no effect on chili callus induction from hypocotyl. The ChC2 (medium containing BA 3 mg/L and IAA 0.5 mg/L) is the best medium for callus induction from both hypocotyl and cotyledon explants. These findings match with the results of (Sripichit and Shigenaga, 1987) who also favored the role of BA over Kin for induction of shoot formation in chili. Agrawal and Chandra (1989) found that IAA and BA produced the best results for shoot bud formation when applied in combination. Out of the test genotypes estimated, only SP developed watery, green fluffy, and morphogenetic calli. Peddaboina and Thamidala (2003) also reported similar observations with other genotypes of *C. annuum* viz., Americano and Dulce Italiano.

Plant regeneration was achieved in 3 chili genotype but with very low plant regeneration frequency. Three different mixtures of regeneration media were tested and a combination of high cytokinin to auxin ratio in the regeneration media was found to be effective for chili plant regeneration (Hegde *et al.*, 2017; İzgü *et al.*, 2020).

Plant regeneration frequency varied between 0-16 percent and maximum shoot regeneration was observed in SP (16.0) genotype calli (Calli obtained from hypocotyl explants), While TP (Calli obtained from both hypocotyl and cotyledon explants) did not show any response to plant regeneration potential. ChSR3 (medium containing BA 3mg/L and IAA 0.05 mg/L) the best medium for plant regeneration from hypocotyl calli but there is no effect of different concentrations of BA (3-5mg/L) on plant regeneration from cotyledon explants. It was thus noted that the hypocotyl explant gave maximum regeneration potential on a low concentration of BA but hypocotyl callus transformed to brownish-black and non-regenerable upon increasing the concentration of BA up to 5 mg/L Regenerated plantlets were rooted in nutrients and hormone free MS medium. 3-4 weeks later, rooted plants were shifted to soil for acclimatization. Plant regeneration of pepper plants via callus is not common due to the problems during callus induction and its development into the plant (Peddaboina and Thamidala, 2006). In the case of other genotypes, the shoot buds either do not elongate or may produce distorted leaves (Valera-Montero, 1992; Ebida, 1993). Some difficulties were experienced in our present study and very low shoot elongation was obtained. Attempts to elongate these shoot buds, such as culture in high BA and low IAA (Peddaboina and Thamidala, 2006) and addition of GA3 or AgNO3 were unsuccessful. In this study, the more callus formation but the low rate of regeneration was found higher that might be attributed to comparatively higher doses of auxin which is used to induce callus in the medium. However, the exact level of hormones in the callus initiation medium needs a compromise between callus induction and regeneration frequency.

Transfer of material from callus induction medium to plant regeneration medium promoted the regeneration capability of the genotype otherwise prolonged culturing on the callus induction medium made callus compact and non-regenerable. So, it is advisable to transfer the material soon after induction of callus to shoot regeneration medium. Dependence of genotype is a critical factor that could impact the organogenesis in chili tissue cultures. As different cultivars of chili have strong genotype specificity for regeneration and are considered critical factors for previously employed regeneration protocols for specific cultivars.

Superficial regeneration of shoots from genetically manipulated cells is among the two strategies used commonly. However, the selection of responsive genotype and explant source is the first strategy while optimization of cultural and environmental conditions for the enhanced genetic potential is the second one. Before deciding the genotype for tissue culture, there should be compared with other genotypes to establish an efficient regeneration system. The logic behind this could be different parts of genotypes could be more adaptive in contrast to former ones while not in the case of others. In our study, the results have shown that SP responded best on hypocotyl explants than cotyledon while TP did not in any combination of hormone used, so TP is the highly recalcitrant genotype. Thus, *C. annuum* genotype SP was found to be the most suitable among the four genotypes studied for subsequent genetic transformation studies. Its calli will be used as recipients of exogenous DNA in genetic manipulation.

Classical plant breeding techniques have been widely used to rise chili yields with improved varieties selection (Baoxi *et al*., 2000; Lee and Hwang, 2003) which are tolerant to abiotic stress Baoxi *et al*., 2000; Lee and Hwang, 2003; Kim *et al*., 2002). Unluckily, some important factors *i.e.,* resistance to herbicides and pathogens, and absence from the genetic pools of chili genotypes (Mijatovic et a., 2005; Kang *et al*., 2007). The use of plant transformation techniques to introduce resistance genes into plant genomes may have a significant effect on the quality and yield of chili. The establishment of a successful transformation system for the chili plant for the regeneration of particular species is a critical step for its transformation.

Development of Chili plant regeneration system

A protocol for chili plant regeneration was developed and among the four genotypes, calli of genotype SP displayed morphogenetic potential and capacity to regenerate complete plantlets. Thus, *C. annuum* genotype SP was found to be the most suitable among the four genotypes were studied for subsequent genetic transformation studies, and SP explants (both hypocotyl and cotyledon) were used as recipients of exogenous DNA in genetic manipulation.

Effect of different factors on Agrobacterium-mediated plant transformation in chili pepper (C. annuum L)

The first most important step to be considered is the optimization of *Agrobacterium*-mediated plant interaction which includes the reliability of the bacterial strain that can develop a localized necrosis process in wounded tissues (Kochieva and Ryzhova, 2009). The effects of explant and different conditions for co-cultivation with *A. tumefaciens*, on the transformation efficiency of SP genotypes, were examined. Explants were inoculated and co-cultured with *Agrobacterium* strains (*A. tumefaciens* LBA4404 having 35S GFP/pFGC construct for 8–10 minutes. *A. tumefaciens* cell inoculate at different temperatures (22 and 25°C), photoperiod (16h light 8h dark and complete darkness), and co-cultivation periods (01, 02 and 03 minutes). Acetosyringone – used as an indicator of *A. tumefaciens vir* genes improve transformation efficiency. Absolute acetosyringone dependency has been observed in *C. annuum* (Kim *et al*., 2001), where acetosyringone was one of the essential components for transformation. However, after inoculating explants with *A. tumefaciens*, the transient expression of the green fluorescent protein (GFP) reporter gene was very low. GFP activity was only exhibited by explants that were inoculated with *A. tumefaciens* culture having acetosyringone. Reporter gene expression generally was lost completely from the tissue 20 days after transformation and calli become dead after 40-45 days.

The problems of the poor survival rate of calli during *Agrobacterium*-mediated transformation were due to hypersensitive response. *In-vitro* recalcitrance of plants has been related to reactive oxygen species (ROS) production (Benson, 2000). Higher levels of free radical activity were found in resistant genotypes of potato and grape as well as in non-embryogenic calli of rice crop (Bailey *et al*., 1994; Benson, 2000). The use of antioxidant silver oxide in sugarcane transformation caused an 80 % cell death which was reduced in comparison to controls, and the quality of the callus was not disturbed in any phase of tissue cul-

ture (Enriquez-Obregon *et al*., 1997). Silver nitrate (2mg/L) in ChC2 selection medium, attempted to reduce ROS response and it also did not affect the transformation efficiency but in the presence of silver nitrate calli remained green and watery up to 50-60 days.The presence of the transgene in the callus was confirmed by PCR using transgene-specific primers. This approach could be used to develop genetic resistance in different pepper cultivars, with the goal of producing pathogen and metabolic engineering resistance.

Conclusions and Recommendations

In present study, we investigate the response of four chili genotypes for callus induction, plant regeneration and plant transformation. The study was conducted to observe the effect of genotypes, culture conditions and growth regulators on plant regeneration of chili pepper (*C. annuum*) genotypes grown in Pakistan including Seedex Pepper (SP), Loungi, Tatapuri, and Sanam. Calli obtained from both hypocotyl and cotyledon explants showed a significant increase in regeneration potential compared to those obtained from non-explanted plants. Plant regeneration frequency varied from (0-16.0) and maximum shoot regeneration was observed in SP (16.2) and TP (17.3) genotypes. Calli obtained from both hypocotyl and cotyledon explants showed maximum regeneration potential. ChSR3 (medium containing BA 3mg/L and IAA 0.05 mg/L) was found to be the best medium for plant regeneration from hypocotyl calli. The hypocotyl explant gave maximum regeneration potential on low levels of BA but became non-generable upon increasing the concentration of BA up to 5 mg/L. Regenerated plantlets were rooted in nutrients and hormone free MS medium. 3-4 weeks later, rooted plants were shifted to soil for acclimatization. For both hypocotyl and cotyledon explants, SP was found to be the most sensitive of the genotypes tested. This procedure could be applied to other cultivars of pepper to induced genetic resistance aiming to produce resistance for pathogen and metabolic engineering.

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Novelty Statement

This research was conducted to evaluate commercial genotypes of chili for callus response and to develop an efficient *in-vitro* clonal propagation protocol. This procedure could be applied to other cultivars of pepper to induce genetic resistance aiming to produce resistance for pathogen and metabolic engineering.

Author's Contribution

Muhammad Shafiq and Sehrish Mushtaq: Carried out research experiments and analyzed data.
Tehseen Ashraf and Naveeda Anjum: Wrote and edited this manuscript, reviewed it and submitted the manuscript.
Muhammad Asim and Muhammad Aqeel Feroze: Provided assistance in manuscript preparation.
Malik Abdur Rehman and Marja Aziz: Revised and improved the manuscript.

Conflict of Interest
The authors declare that they have no conflict of interest.

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