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PLANT BIOTECHNOLOGY | ORIGINAL ARTICLE

In vitro regeneration of hybrid squash (*Cucurbita pepo* L.) cv. First Runner from cotyledonary node explants

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ABSTRACT

Squash (*Cucurbita pepo* L.) is highly polymorphic in nature which could contribute its different regeneration potentiality for different genotypes. The present investigation was carried to know the effects of plant growth regulators, explant types, explants age and $AgNO_3$ on *in vitro* regeneration potentiality of hybrid squash genotype and to optimize regeneration protocol. In this protocol, cotyledon and hypocotyl segments were grown on MS medium supplemented with 1.5 mg L^{-1} 6-Benzylaminopurine (BA) and 0.2 mg L^{-1} α-Naphthalene acetic acid (NAA), successfully induced callus and subsequently shoots for *C. pepo* L. cv. First Runner. The height percentage of shoot production frequency was 66.7% from 6 days old cotyledon explants in MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA combination. The shoot regeneration frequency was increased up to 86.67% when 2.0 mg L^{-1} $AgNO_3$ was added with MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA media combination by using 5 days old cotyledon explants. Shoot regeneration potentiality of four other *C. pepo* genotypes were also investigated with this system and showed a substantial amount of regeneration potentiality for different genotypes. Regenerated shoots induced the height 93.33% rooting frequency which promoted long, thick roots on 10 to 12 days in MS medium supplemented with 0.1 mg L^{-1} NAA. The regenerated plantlets with long and thick roots were acclimatized in pot soil and eventually grown in natural environment with culture facility. The present study describes a simple and efficient protocol for *in vitro* plant regeneration of hybrid *C. pepo* genotypes and may be utilized for further transgenic development.

Keywords: Squash, *in vitro* regeneration, cotyledon, hypocotyl, growth factors

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1 Introduction

Squash (*Cucurbita pepo* L.) is an important crop under the world largest vegetable family, Cucurbitaceae. Squash consumption is becoming popular due to its innate nutritional value such as rich in vitamins and minerals with adequate fibres and carbohydrates (Elinge et al., 2012; Saavedra et al., 2013; Adelerin et al., 2022). It has also great medicinal value incurring valuable compounds of flavonoids and antioxidative substances (Itle and Kabelka, 2009; Kostecka-Gugała et al., 2020) and physiological functions like antidiabetic, anti-hypercholesterolemic, anti-inflammatory and antihypertensive activity etc. (Adams et al., 2011). Since the independence of Bangladesh in 1971, the acreage and production of Cucurbita species (squash, pumpkin and gourd) were

increased almost 5 times, yielded 0.67 million tons in 2020 using 0.061 million hectares of land (FAOSTAT, 2022). Comparing the global production of 27.96 million tons in 2020, Bangladesh contributed only 2.4% which is very subtle amount than the other neighboring countries China and India, yielded 26.76% and 18.29%, respectively (FAOSTAT, 2022). And having almost similar environment in India, there is a great scope to increase both acreage and production of Cucurbita species in Bangladesh. Thus, it is crucial need to improve squash cultivar to meet the requirement of vegetable for ensuring global food security.

The diverse topography and edaphic features of Bangladesh can accelerate the crop diversification and production (Azad, 2021). Squash can grow well in any types of soil, even in unproductive fellow and marginal lands (Santosh et al., 2017). In Bangladesh, usually squash is cultivated in the summer season in which insect, pest and disease infestations are severer (Khan et al., 2021), resulting the yield loss. Especially, major types of viral diseases viz. cucumber mosaic virus, zucchini yellow mosaic virus, watermelon mosaic virus and papaya ringspot virus (Sydanmetsa and Mbanzibwa, 2016) as well as cucurbit fruit fly infestation (Gautam et al., 2021) in squash have been reported earlier. Development of resistant transgenic cultivars against such viruses and insects is a prerequisite for successful squash production. Regarding this, tissue culture technique is a prerequisite to develop resistant transgenic cultivars to overcome these problems.

The improvement of squash through traditional breeding is little challenging because of its narrower genetic base, lower genetic variability as well as assorted crossing problems with their relevant species (Miguel, 2021). And regeneration via organogenesis over the conventional breeding privilege to produce plant from seedless genotype (Yildiz, 2012), preserve endangered species as well as speedy production of seasonal and weather dependent plants (García-Gonzáles et al., 2010). Squash is highly polymorphic in nature, shows the diverse potentiality in tissue culture even in the same cultivar (Kiss-Baba et al., 2006). Regeneration through somatic embryogenesis in *C. pepo* have been reported earlier (Chee, 1992; Gonsalves et al., 1995) which required long time, however, regeneration using cotyledon with attached hypocotyl is an alternative protocols provoked less time also been addressed (Stipp et al., 2012; Mookkan, 2015).

Regeneration efficiency in tissue culture depends on different factors like genotypes, plant growth regulators, explant types and age, culture medium as well as incubation condition (Nanasato et al., 2013; Grozeva and Velkov, 2014; Miguel, 2021). And optimization of regeneration system in *C. pepo* genotypes using cotyledon and hypocotyl grown in MS medium with different concentration of Benzylaminopurine (BA) and α-Naphthalene acetic acid (NAA) induced callus and subsequently shoots have also been reported in zucchini squash (Stipp et al., 2012; Mookkan, 2015). Moreover, different explant types expressed differently in both embryogenesis and organogenesis system of squash tissue culture (Obembe et al., 2017; Dursun et al., 2019). Some other factors like explant age also influenced in plantlets regeneration potentiality of squash (Lee et al., 2003; Stipp et al., 2012; Nanasato et al., 2013). However, silver nitrate ($AgNO_3$) has evidenced to be a very effective inhibitor of ethylene action and is widely used in plant tissue culture of *C. sativus* (Venkatachalam et al., 2018) but limitedly reported for *C. pepo*. Keeping all these in mind, in this present study, genetically diverse and superior squash cultivar-First Runner (Sajid et al., 2022), a widely used cultivar in Bangladesh is used for the *in vitro* regeneration. However, this cultivar is highly susceptible to fruit fly and can cause damage up to even in 100% (Sajid et al., 2022) and is crucial need to develop fruit fly resistant squash genotypes. Therefore, the study was investigated to develop an efficient regeneration protocol using different growth factors from cotyledonary node explants of important squash cultivar-First Runner.

2 Materials and Methods

2.1 Location

The experiment was carried out during the period of July 2020 to June 2021 in the Genetic Engineering Laboratory under the Department of Genetics and Plant Breeding, Sylhet Agricultural University, Bangladesh.

2.2 Plant material

The seeds of five hybrid squash (*Cucurbita pepo* L.) genotypes namely First Runner, Runner, Alaska, Blossom house and Balam house were collected from local market to fulfil the objectives of the present study. Among these varieties First Runner was used to standardize the plant regeneration protocol of hybrid *C. pepo* genotype and other genotypes were used to observe their plantlet regeneration potentiality.

2.3 Sterilization and seed germination

The seeds were soaked for 2-3 hours in tap water, and then the seed coats were removed with forceps in laminar air flow cabinet. The pealed seeds were then sterilized for 2 min in 70% ethanol (MERCK, Germany), followed by a 10-min immersion in a 10% Clorox (Sodium hypochlorite, The Clorox Company, Oakland, USA). The seeds were then washed in sterile distilled water for four times. Two seeds were placed per germination medium comprising half strength MS (Murashige and Skoog, 1962) medium (Duchefa,

Netherland) supplemented with 3% sucrose (SMART-LAB, Indonesia) and 1% agar (MERCK, Germany). Seeds were germinated in a growth chamber (GC-300TL, JEIO TECH, Korea) maintained at 25 °C/23 °C (day/night) temperature under a 16/8 hours (day/night) photoperiod for 4 to 8 days with light supplied by cool-white fluorescent lamps at an intensity of 100 mmol m^{-2} per second (culture condition).

2.4 Explant preparation

Six days old seedlings were used to prepare cotyledon and hypocotyl explants. Cotyledons alone with 0.5-1.0 mm petioles were excised very carefully from the hypocotyl. Then the cotyledon was cut in half and the distal parts discarded. The hypocotyls were then isolated from the root tip and cut into 4.0-5.0 mm length segments.

2.5 Culture media for callus induction

The explants were cultured on MS (Murashige and Skoog, 1962) media supplemented with different concentrations of 6-Benzylaminopurine (BA) (99%, Duchefa Biochemie, the Netherlands) (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}) and α-Naphthaleneacetic acid (NAA) (98%, Duchefa Biochemie, the Netherlands) (0.2, 0.3 and 0.5 mg L^{-1}) to determine optimal medium for callus induction. Ten explants were placed on each culture vessels containing 50 mL callus induction media. Cotyledons along with petioles were placed in upward direction with the petiole in contact with the media whereas hypocotyl segments were placed horizontally on the surface of the media. The culture vessels were sealed with parafilm and marked with permanent marker to indicate specific treatment and incubated in culture condition. The whole procedure was carried out in laminar airflow cabinet. After 15 days of incubation, data were taken on the callus initiation frequency.

2.6 Culture media for shoot regeneration and subculture

After 15 days of incubation, the calli developed from the explants and attained a convenient size. The induced celli were transferred into same fresh media for shoot development in an embryogenic nature. Shoot induction media comprised MS salts and vitamins, 3% sucrose, 1% agar and various concentrations of BA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}) and NAA (0.2, 0.3 and 0.5 mg L^{-1}). Watery, spongy, very compact, brown and dead portions of calli were discarded during each subculture. Friable, nodular calli were assumed have the potentially of embryogenesis and were selected for maintenance and regeneration. In addition, various concentrations of $AgNO_3$ (1.0, 2.0, 3.0, 4.0 and 5.0 mg L^{-1}) were used along with the best combination of previously used shoot regeneration media to investigate their effect on shoot regeneration. In vitro regenerated shoots were sub-cultured regularly to fresh media at an interval of 10-12 days for further multiplication.

2.7 Rooting and acclimatization

When regenerated shoots were attained a height of 2-3 cm they were excised and transferred to half strength MS media supplemented with different concentrations of NAA (0, 0.1, 0.2 and 0.5 mg L^{-1}) for rooting (single plantlet per vial with 3 replications). Every time, the cultured vessels were sealed with parafilm and marked with a permanent marker to indicate each treatment and were incubated in growth chamber at culture condition. Three to four cm in length of plantlets with sufficient root system were taken out carefully from the culture vessels and washed gently in tap water to remove agar medium and sucrose trace elements to discourage infection by fungal contamination. The plantlets were then transplanted to moistened soil in pots and covered with glassware (beaker) for preventing desiccation. After proper hardening, the plantlets were transferred to natural environment.

2.8 Statistical analysis

The experiment was arranged in completely randomized design (CRD) with three replications. The recorded data for different parameters were statistically analysed to ascertain the significance of the experimental results. The mean and standard deviation for all treatments were calculated by using MS Excel 2010. The significance and difference between means were evaluated by Dunkan's Multiple Range Test (DMRT) using R statistical package (R Core Team, 2021).

3 Results

3.1 Callus induction

In this experiment, MS media supplemented with various concentrations of 6-Benzylaminopurine (BA) (0.5 to 3.0 mg L^{-1}) and α-Naphthalene acetic acid (NAA) (0.2 to 0.5 mg L^{-1}) were used to optimize the media for callus induction from cotyledon and hypocotyl explants in squash (*Cucurbita pepo* L.) cv. First Runner (Fig. 1). A total of 25 different media combinations were tested, in which cotyledon explants showed the highest (86.7%) callus initiation frequency in MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA combination while the lowest (16.7%) was found in MS + 3.0 mg L^{-1} BA and MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA combinations. In case of hypocotyl explants, the highest (76.7%) callus initiation frequency was obtained in MS + 1.5

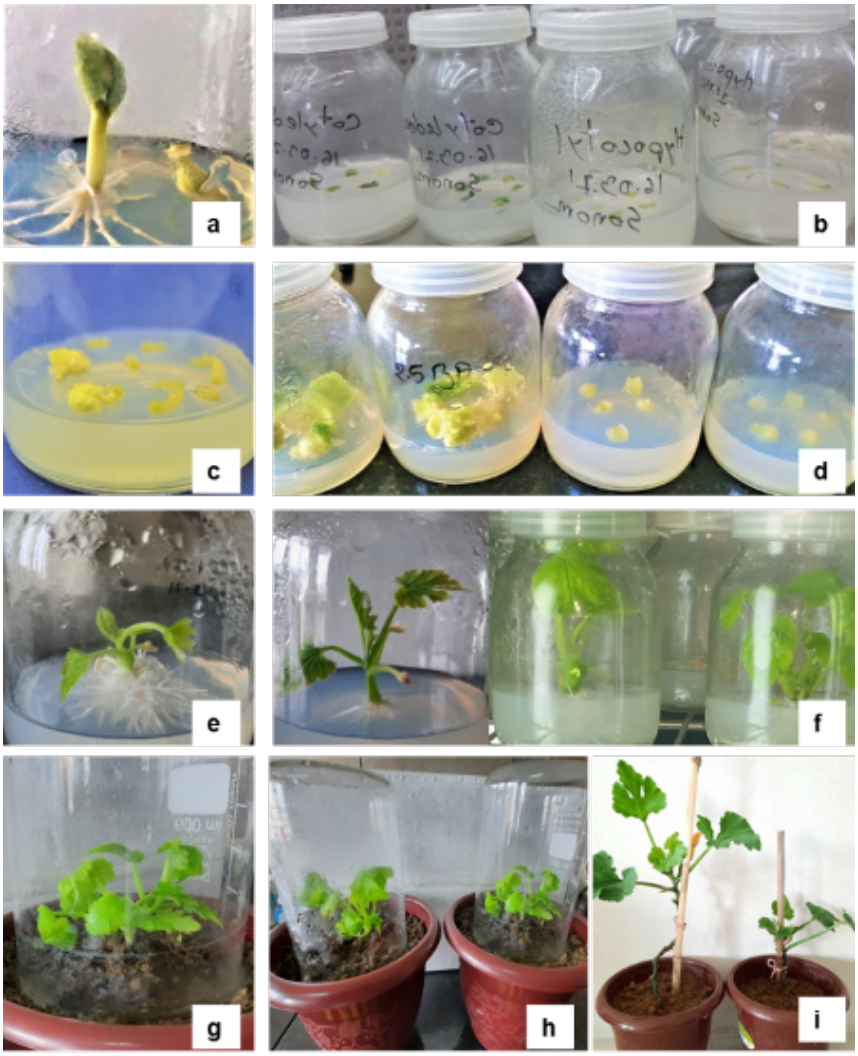

Figure 1. The *in vitro* regeneration process of squash (*Cucurbita pepo* L.) cv. First Runner. (a) 6 days old seedlings grown in MS media, (b) cotyledon and hypocotyl explants on callus induction medium (MS + 1.5 mg L^{-1}BA + 0.2 mg L^{-1} NAA) at first day of culture, (c) fifteen days old callus obtained from hypocotyl explants in callus induction medium, (d) fifteen days old callus obtained from cotyledon and hypocotyl explants in callus induction medium, (e) shoot initiation in shoot induction medium (MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA), (f) initiation of roots in MS + 0.1 mg L^{-1} NAA medium, (g & h) acclimatized plant in soil, (i) flowered squash plant in natural environment

mg L^{-1} BA + 0.2 mg L^{-1} NAA combination and the lowest (13.3%) was attained in MS + 3.0 mg L^{-1} BA combination (Table 1). A significant difference was found in callus initiation frequency between cotyledon and hypocotyl explants and the cotyledon explants showed better performance than the hypocotyl explants. Hormone free MS basal medium (control treatment) did not produce any callus and died after a few days of culture (Table 1).

3.2 Shoot regeneration

A significant variation in the shoot regeneration was found on media containing different concentrations of BA and NAA. Shoot bud formation was started from the callus after two weeks of incubation in MS media supplemented with various concentrations of BA (0.5 to 3.0 mg L^{-1}) and NAA (0.2 to 0.5 mg L^{-1}). Among the combinations, MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA showed the highest 66.7% and 60.0% shoot regeneration frequency from cotyledon and hypocotyl explant, respectively (Table 1). The lowest frequency of shoot regeneration (6.7%) was observed in MS + 3.0 mg L^{-1} BA for both cotyledon and hypocotyl explants. However, both the cotyledon and hypocotyl explants did not produce any shoot in control (MS basal), MS + 2.5 mg L^{-1} BA + 0.5 mg L^{-1} NAA and MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA media combinations (Table 2). This result indicated that, high BA and NAA ratio is not suitable combination for *in vitro* shoot bud formation in squash (Table 1).

Table 1. Frequencies of callus initiation and shoot regeneration from 6 days old cotyledon and hypocotyl explants of squash (*Cucurbita pepo*) cv. First Runner on MS media supplemented with various concentrations of BA and NAA

Treatments	Callus initiation frequency (%)		Shoot regeneration frequency (%)	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
MS	0.0±0.0	0.0±0.0	0.0±0.0f	0.0±0.0e
MS + 0.5 BA	30.0±1.0ij	26.7±0.6ijk	13.3±0.6def	13.3±0.6cde
MS + 1.0 BA	40.0±1.0hi	33.3±0.6hij	26.7±1.2bcdef	20.0±1.0bcde
MS + 1.5 BA	50.0±1.0fgh	43.3±0.6efgh	46.7±1.2abc	33.3±1.2abcd
MS + 2.0 BA	40.0±0.0hi	40.0±0.0fgh	26.7±0.6bcdef	26.7±0.6bcde
MS + 2.5 BA	26.7±0.6jk	23.3±0.6jkl	20.0±0.0cdef	20.0±1.0bcde
MS + 3.0 BA	16.7±0.6k	13.3±0.6l	6.7±0.6ef	6.7±0.6de
MS + o.5 BA + 0.2 NAA	56.7±0.6def	53.3±0.6cde	33.3±1.2bcde	26.7±0.6bcde
MS + 1.0 BA + 0.2 NAA	66.7±0.6bcd	63.3±0.6bc	46.7±1.5abc	46.7±0.6ab
MS + 1.5 BA + 0.2 NAA	86.7±0.6a	76.7±0.6a	66.7±0.6a	60.0±1.0a
MS + 2.0 BA + 0.2 NAA	76.7±0.6ab	66.7±0.6ab	53.3±0.6ab	46.7±0.6ab
MS + 2.5 BA + 0.2 NAA	56.7±0.6def	50.0±1.0def	40.0±0.0abcd	33.3±1.2abcd
MS + 3.0 BA + 0.2 NAA	40.0±1.0hi	36.7±0.6ghi	20.0±1.0cdef	13.3±1.2cde
MS + 0.5 BA + 0.3 NAA	46.7±0.6fgh	40.0±1.0fgh	20.0±1.0cdef	26.7±0.6bcde
MS + 1.0 BA + 0.3 NAA	56.7±0.6def	46.7±0.6defg	40.0±1.0	40.0±0.0abc
MS + 1.5 BA + 0.3 NAA	73.3±0.6bc	66.7±0.6ab	46.7±0.6ab	46.7±0.6ab
MS + 2.0 BA + 0.3 NAA	63.3±0.6cde	56.7±1.2bcd	33.3±1.2bcde	26.7±0.6bcde
MS + 2.5 BA + 0.3 NAA	50.0±1.0fgh	43.3±0.6efgh	26.7±0.6bcdef	13.3±1.2cde
MS + 3.0 BA + 0.3 NAA	26.7±0.6jk	20.0±1.0kl	13.3±0.6def	6.7±0.6de
MS + 0.5 BA + 0.5 NAA	43.3±0.6gh	36.7±0.6ghi	13.3±1.2def	20.0±0.0bcde
MS + 1.0 BA + 0.5 NAA	50.0±1.0fgh	43.3±0.6efgh	26.7±1.2bcdef	26.7±0.6bcde
MS + 1.5 BA + 0.5 NAA	53.3±0.6efg	40.0±1.0fgh	33.3±1.2bcde	33.3±2.1abcd
MS + 2.0 BA + 0.5 NAA	43.3±0.6gh	40.0±1.0fgh	26.7±0.6bcdef	13.3±0.6cde
MS + 2.5 BA + 0.5 NAA	30.0±1.0ij	26.7±0.6ijk	0.0±0.0f	0.0±0.0e
MS + 3.0 BA + 0.5 NAA	16.7±0.6k	16.7±0.6kl	0.0±0.0f	0.0±0.0e

Data consist of three replicates, each comprising 10 explants. The mean values were compared by DMRT. Mean ± SD followed by same letters are not significantly different at P = 0.05

3.3 Effect of explant age

In order to investigate the effect of explants age on shoot regeneration of squash, the cotyledon explants of different ages (4 to 8 days old) were cultured on the best callus induction medium(MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA) and subsequently best shoot regeneration medium(MS + 1.0 mg L^{-1} BA + 0.2 mg L^{-1} NAA). Explants from 1 to 3 days old seedling were too small and were not used in this experiment. Cotyledon explants of 5 days old seedlings showed the highest (73.3%) shoot regeneration frequency followed by 6 days old explants (66.7%) whereas explants of 8 days old seedlings showed the lowest (33.3%) shoot regeneration frequency after three weeks of explant incubation (Fig. 2).

3.4 Influence of $AgNO_3$

In this experiment, the effect of $AgNO_3$ on shoot regeneration in squash was also investigated. Five days old cotyledon explants of squash (*C. pepo* L.) cv. Firsr Runner were cultured on best shoot regeneration medium (MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA) together with different concentrations of $AgNO_3$ (1.0, 2.0, 3.0, 4.0 and 5.0 mg L^{-1}) (Fig. 3). The highest (86.67%) shoot regeneration frequency was observed in shoot regeneration media supplemented with 2.0 mg L^{-1} $AgNO_3$ whereas, the lowest (40.0%) shoot regeneration frequency was observed in shoot regeneration medium supplemented with 5.0 mg L^{-1} $AgNO_3$.

3.5 Genotypic variation

Five days old cotyledon explants of five different *C. pepo* genotypes namely First Runner, Runner, Alaska, Blossom house and Balam house were cultured on shoot regeneration medium (MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA) in addition to 2 mg L^{-1} $AgNO_3$ to investigate their shoot regeneration potentially. Shoot regeneration frequency was 86.67%, 73.33%, 40.00%, 60.00% and 53.33% in First Runner, Runner, Alaska, Blossom house and Balam house respectively (Fig. 4).

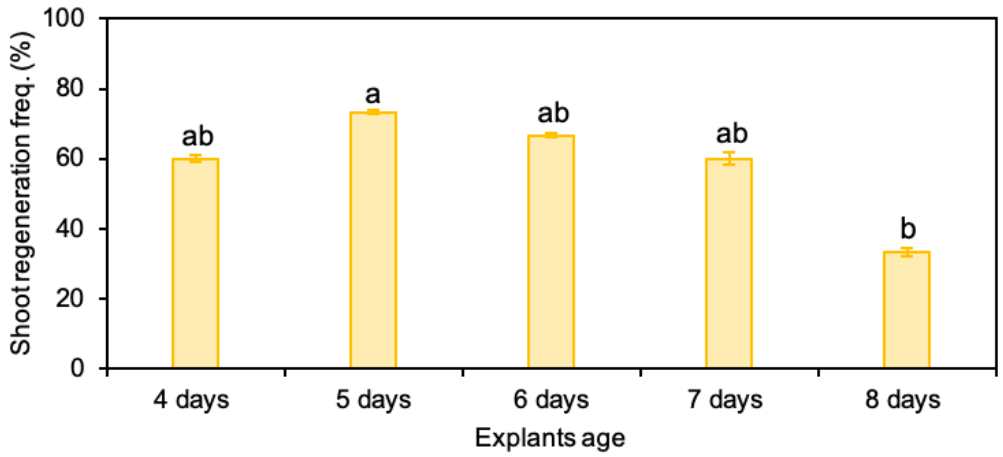

Figure 2. Effect of explant age on shoot regeneration from cotyledon explants of squash (*Cucurbita pepo* L.) cv. First Runner. Data consist of three replications and 5 cotyledon explants were used for each replication. Bars represent SD of means. Values with different letters are significantly different at P value = 0.05 (DMRT)

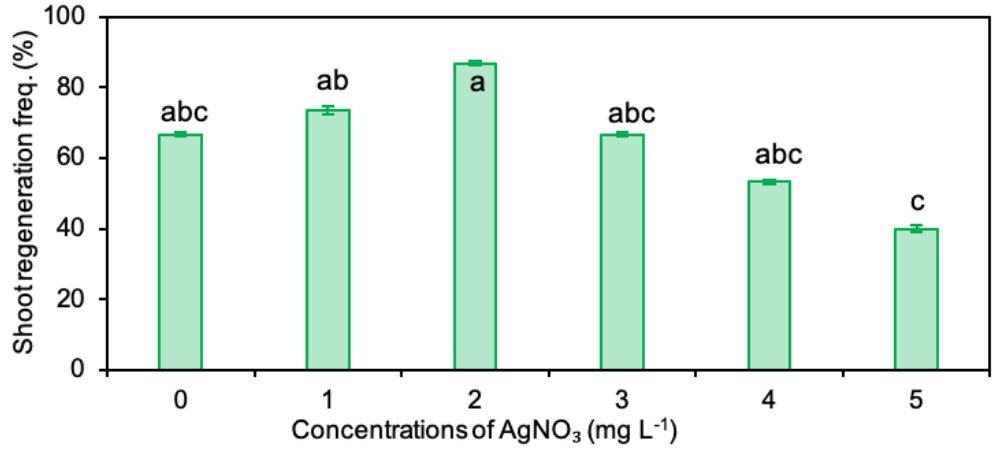

Figure 3. Effect of $AgNO_3$ concentrations on shoot regeneration from 5 days old cotyledon explants of squash (*Cucurbita pepo* L.) cv. First Runner. Data consist of three replications and 5 cotyledon explants were used for each replication. Bars represent SD of the means. Values with different letters are significantly different at P = 0.05 (DMRT)

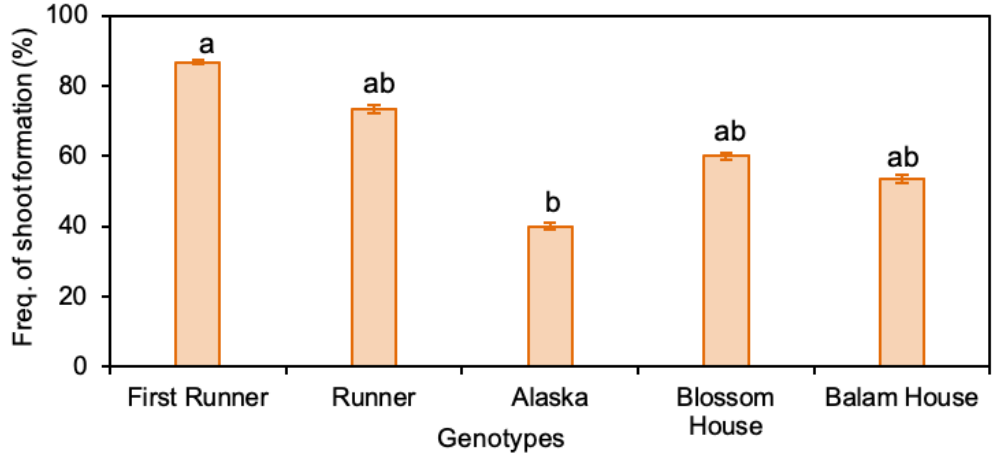

Figure 4. Influence of genotypes on shoot regeneration from 5 days old cotyledon explants of squash (*Cucurbita pepo* L.). Data consist of three replications and 5 explants were used for each replication. Bars represent SD of means. Values with different letters are significantly different at P = 0.05 (DMRT)

3.6 Root induction

At the final stage of *in vitro* plant development, 2-3 cm elongated shoots were excised out and transferred to rooting media, MS medium supplemented with different concentration of NAA (0, 0.1, 0.2, 0.5 and 1.0 mg L^{-1}). Among the five tested media, the maximum root formation (93.33%) was observed in MS + 0.1 mg L^{-1} NAA medium whereas the lowest rooting frequency (46.67%) was observed in MS + 1.0 mg L^{-1} NAA MS medium (Fig. 5). Within 6 days of culture, root formation started and plantlets produced well developed root system within 10 to 12 days of incubation.

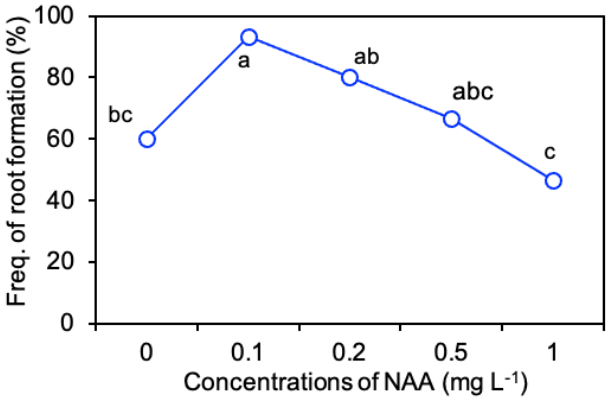

Figure 5. Influence of genotypes on shoot regeneration from 5 days old cotyledon explants of squash (*Cucurbita pepo* L.). Data consist of three replications and 5 explants were used for each replication. Bars represent SD of means. Values with different letters are significantly different at P = 0.05 (DMRT)

4 Discussion

Squash (*Cucurbita pepo* L.) is highly polymorphic vegetable species in nature under cucurbitaceae family with major importance worldwide (Kathiravan et al., 2006). In this research several factors of somatic embryogenesis of hybrid *C. pepo* genotypes were studied. Different genotypes of *C. pepo* have been regenerated via somatic embryogenesis (Chee, 1992; Gonsalves et al., 1995; Urbanek et al., 2004) and organogenesis (Lee et al., 2003; Kiss-Baba et al., 2006; Stipp et al., 2012; Mookkan, 2015; Obembe et al., 2017; Dursun et al., 2019) to date. A wide range of genetic variation within *C. pepo* species could contribute different regeneration potentiality for different genotypes (Gonsalves et al., 1995; Kathiravan et al., 2006; Stipp et al., 2012).

In this investigation, cotyledon and hypocotyl explants (excised from *in vitro* grown seedlings) were used to find out their callusing ability. Both the explants produced compact and friable greenish white or brownish in color calli with very few watery and pale brown in color calli (Fig. 1c & Fig. 1d). Cotyledon explants were found more responsive than the hypocotyl explants in both callus formation and subsequent shoot initiation. This result can be favorably compared with the result of *Cucumis melo* L. (Souza et al., 2006) and *C. pepo* L. (Obembe et al., 2017; Dursun et al., 2019) that the cotyledonary explants performed then the other explants like hypocotyl. One of the reasons for this finding might be that cotyledons have a remarkable morphogenetic potentiality than hypocotyl for callus induction. The hormone free MS medium did not produce any callus and explants were died after few days of culture. Considering only BA concentration, cotyledon and hypocotyl explants were produced the maximum 50.0% and 43.3% callus, respectively in MS + 1.5 mg L^{-1} BA concentration. Whereas, BA and NAA combinations were produced the maximum 86.7% and 76.7% callus formation frequency for cotyledon and hypocotyl explants respectively (Table 1). These results indicated that the combinations of BA with NAA gave the better result than BA alone in case of callus initiation of *C. pepo* cv. First Runner. Considering two plant growth regulators (BA and NAA), the BA concentrations were mostly regulated the callus and shoot formation frequency rather the concentration of NAA. The callus initiation frequency was increased with the increase of BA concentrations up to 1.5 mg L^{-1} then it declines when the concentration of NAA remain same for both the cotyledon and hypocotyl explants (Table 1). However, the present findings of callus formation (86.7%) were higher than the findings of Miguel (2021) reported 72.65% and 70% callus formation frequency for cotyledon explants.

Cytokinens are the plant hormones mainly regulated the cell division and differentiation for adventitious shoot bud formation and development from callus (Deb et al., 2019). These compounds form lateral shoot buds by overcoming apical shoot bud formation dormancy while callus are placed to shoot regeneration media (George et al., 2007). In the present investigation, compact and friable greenish white or brownish in colour callus were sub-cultured for shoot regeneration. Although, shoot induction varies with the proportion of plant growth regulators and explants type in (Trujillo-Moya and Gisbert, 2012) and *Solanum sisymbriifolium* (Deb et al., 2019), the present investigation have found the maximum shoot induction frequency in the same media combination produced the height number of callus (MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA). Additionally, similar as callus initiation, cotyledon explant showed better shoot regeneration frequency than the hypocotyl explant. This result can be compliant with the results of Gonsalves et al. (1995) stated that, cotyledons are the most efficient and dependable source of somatic embryogenesis. Additionally, most of the previous organogen-

esis protocols of squash (Lee et al., 2003; Kiss-Bába et al., 2010; Stipp et al., 2012; Obembe et al., 2017; Dursun et al., 2019) have been developed using cotyledon explant. However, it was found that the height 66.7% shoot regeneration frequency obtained from 6 days cotyledon explants. But the regeneration frequency was increased up to 73.3% while using 5 days old cotyledon explants. A steady decrease in shoot regeneration frequency was observed in the explants derived from 5 to 8 days old seedlings. So, the age of explants used for *in vitro* regeneration is an important factor for the regeneration of *C. pepo* cv. First Runner. Some other previous finding of (*Cucumis sativus*) (Kim et al., 2000), watermelon (*Citrullus lanatus*) (Compton, 2000), winter squash (*Cucurbita maxima*) (Lee et al., 2003) and summer squash (*C. pepo* L.) (Stipp et al., 2012) were also in an agreement with this result that, the explants collected from the seedling older than 4-5 days old produce decline rate of shoot for cucumber.

As like explant age the present investigation also found the influence of $AgNO_3$ on shoot formation. A maximum of 86.67% shoot induction frequency was observed by adding 2.0 mg L^{-1} $AgNO_3$ in the MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA (shoot induction medium). The shoot regeneration via somatic embryogenesis of *C. pepo* cv. First Runner also influenced by $AgNO_3$ like some other species *Brassica campestris* (Sarker et al., 2016). Moreover, Venkatachalam et al. (2018) reported that, the addition of $AgNO_3$ in shoot regeneration medium enhanced the rate of shoot bud formation frequency *via* organogenesis in *C. sativus*. However, to check the regeneration potentiality of the proposed media combination (MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA + 2.0 mg L^{-1} $AgNO_3$) was tested with other four squash genotypes (Runner, Alaska, Blossom house and Balam house). Shoot regeneration potentiality was 73.33%, 40.0%, 60.0% and 53.33% in Runner, Alaska, Blossom house and Balam house respectively. A wide range of agro-morphological variations among these varieties (Sajid et al., 2022) could lead different regeneration potentiality for them (Kathiravan et al., 2006).

Generally, lower cytokinin to auxin ratio induces roots with few shoots (Deb et al., 2019). For rooting, only NAA (auxin) in different concentrations (0.1 to 1 mg L^{-1}) were used in this experiment. Results indicated that, NAA free basal MS medium also produced a considerable amount (60%) of roots. MS media itself contained different macro and micro nutrient concentrations which promotes a considerable amount of rooting in regenerated shoots. However, the highest root initiation frequency (93.33%) was observed in MS + 0.1 mg L^{-1} NAA combination. With the increase of NAA concentrations rooting frequency was gradually decreased and the lowest (46.67%) was observed at 1.0 mg L^{-1} NAA concentration.

5 Conclusion

There is a great impact of explant age and $AgNO_3$ in the *in vitro* regeneration of *Cucurbita pepo* cv. First Runner along with plant growth regulators BA and NAA. However, cotyledon explants excised from 5 days old seedling showed the maximum 86.67% shoot regeneration frequency in MS + 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA + 2.0 mg L^{-1} $AgNO_3$ combination. Additionally, MS + 0.1 mg L^{-1} NAA combination produced the height rooting frequency. Although, high morphogenetic diversity of *C. pepo* genotypes contributes different regeneration potentiality, this protocol showed a substantial amount of regeneration potentiality for different genotypes. So, this protocol could be utilized for further *in vitro* improvement program of *C. pepo* genotypes.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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