Optimization of important factors for efficient embryogenesis in sweet pepper (*Capsicum annuum*var.*grossum*L.) using anther culture technique

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ABSTRACT

Different combinations of factors affecting androgenic embryo induction in four sweet pepper hybrids, namely Asha, Sympathy, Namelite, and Indra were investigated in this present study. There were three combinations of different factors which were employed such as cold pre-treatment with heat shock, sucrose with activated charcoal, and auxin with cytokine. The experimental results revealed that the highest number of embryos (39.3) per 300 anthers were recorded in 'Asha' (V_1) when buds were cold pre-treated at 4°C for 24 hours (C_2) followed by keeping anthers at 35°C in the dark for 12 days +25°C-Cont. (HS_3). The combination of sucrose with activated charcoal gave the highest number of embryos (48.7) in 'Asha' (V_1), when anthers were excised and placed on CP induction media supplemented with 4% (S_3) sucrose and 0.50 g/l (AC_2) activated charcoal. The combination of 2,4-D with Kinetin revealed the highest number of embryos (43.0) in 'Asha' (V_1)where anthers were excised and placed on media supplemented with 0.50 mg/l of 2,4-D (A_2) with 4.0 mg/l of Kinetin (K_4). An additional experiment was performed by combining the best treatments from the previous experiments, and the highest frequency of embryo induction (23.1%) was observed in 'Asha' (V_1). The embryo formation was primarily observed to be genotype-dependent, with Asha being the most responsive in every set of combinations studied, and Sympathy (V_2) being the least responsive. Aside from genotype dependence, factors, such as cold pre-treatment, heat shock, activated charcoal, carbon source, and plant growth regulators play an important role in embryo induction.

Keywords: *Capsicum annuum*var.*grossum*, anther culture, embryo induction, genotype effect.

DOINumber:10.14704/nq.2022.20.8.NQ44631 **NeuroQuantology2022;20(8):6051-6061**

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Introduction

Sweet pepper or bell pepper (*Capsicum annuum* var. *grossum* L.) is one of the most important vegetables in the world due to its nutritional properties and pharmacological applications, as it contains vitamin C and

numerous carotenoids (Irikova et al., 2011). To meet the market's expanding demand for sweet pepper, new improved cultivars need to be developed so as to increase productivity. To develop true breeding lines, cultivars have traditionally been developed through backcross breeding, followed by self-pollination for several generations. This was labour-intensive and time-consuming. Using Doubled halpoid technology, it is possible to achieve homozygous lines in one generation which is an important criterion for hybrid production. Androgenesis is the technique used to generate the homozygous lines required for hybrid development, followed by chromosome doubling to generate inbred lines. This technique speed up the pathway for the development of new sweet pepper varieties and inbred line development programme having desirable properties that can be used for further breeding (Koleva-Gudeva, 2007; Kim et al., 2004). Wang et al. (1973) and George and Narayanaswamy (1973) reported plant regeneration from *C. annuum* anthers for the first time. Following that, several other researchers published studies on various aspects of androgenesis, including anther, shed microspore, and isolated microspore cultures (Sibi et al., 1979; Dumas de Vaulx et al., 1981; Supena et al., 2006a; Kim et al., 2008). Among the three methods, anther culture is the foremost widely utilized for DH production in pepper. In the past studies, it has been observed that several factors affect the frequency of androgenesis. In the androgenesis, the genotype is the most regular limiting factor as it cannot be overcome by donor growing conditions and other culture conditions (Kristiansen and Andersen, 1993;Wang and Zhang, 2001;Rodeva et al., 2004, Buyukalaca et al., 2004;Koleva-Gudeva et al., 2007). In addition, several other factors which affect androgenesis are the developmental stage of microspores, vitality of microspores, anther pre-treatment, medium combinations, culture conditions,carbon source, activated charcoal(Ciner and Tipirdamaz, 2002; Novaczyk and Kisiala, 2006). The purpose of this study was to determine the appropriate combination of factors that directly influence embryogenesis when cultured only on CP induction medium (Dumas de Vaulx et al., 1981).

MATERIALS AND METHODS

Four sweet pepper hybrid genotypes, namely Asha (V_1), Sympathy (V_2), Namelite (V_3), andIndra (V_4) were employed to carry out the anther culture experiment. The donor plants were grown and nurtured in a greenhouse having photoperiod of 16 hours, day and night temperatures of 25–30°C and 15–20°C, respectively at Temperate Breeding and Research Station, ACSEN HYVEG Pvt. Ltd. situated at Kullu (Himachal Pradesh, India) duringFebruary 2020. Buds for experimentation were taken from the first flush of flowering in May 2020.

Flower buds of four varying sizesi.e., 4.75 mm, 5.0 mm, 5.25 mm, and 5.50 mm were used for ascertain of developmental stage and vitality determination.Staining was done for each size using DAPI and FDA-PI for development stage and vitality, respectively. Slides were examined under a staining microscope. The bud size having microspores at late uninucleate to early binucleate stage with maximum vitality (70:30) were selected for the experiment (Rodeva et al., 2007; Parra-Vega et al., 2013).In order to correlate the cold shock effect, half of the selected buds of each entry were kept in the refrigerator at 4°C for 0, 24, and 48 hrs.Buds were surface-sterilized with 2-3 drops of Tween 20 for 15 min, 70% ethanol for one minute, 4% sodium hypochlorite solution for 10 minutes, and then rinsed three times in sterile water before experimentation.Anther culture was performed in threesets of experiments using different treatment combinations. Each treatment was replicated thrice containing 300 anthers per replication. In each experiment, besides the factors being tested, all other experimental conditions and media (CP) werekept same asgiven by Dumas de Vaulx et al., 1981.Observations for embryo induction in each experiment were done on a routine basis and embryos were counted. After analysis, one combined experiment was designed by taking the best results from previous three experiments in two replicates containing 150 anthers per replicate. Data

*e*ISSN1303-5150

wereanalysed for the number of embryos as well as embryo induction frequency. The experimental data were subjected to analysis of variance (ANOVA) using OPSTAT software and to Duncan's multiple range tests with SPSS-16 program for Windows.

To establish the relationship between different cold pre-treatment durations with effective heat shock duration, the anthers from cold pre-treated buds which were kept at 4°C for 0 (C_1),24 (C_2), and 48 (C_3) hours were excised and cultured on 90 mm plates containing CP medium. These cultured plates were kept at 35°C in the dark for 6 (HS_1), 8 (HS_2), 10 (HS_3), and 12 (HS_4) days for heat shock. After the respective days of heat shock, these plates were transferred to 25°C with 16:8 hr photoperiod (Dumas de Vaulx et al., 1981).

To establish the relationship between different sucrose and charcoal concentrations, the CP medium was supplemented with three concentrations of sucrose i.e., 2 (S_1), 3 (S_2), and 4 % (S_3) along with four concentrations of activated charcoal i.e.,0.25 (AC_1), 0.50 (AC_2), 0.75(AC_3) and 1.0 (AC_4) g/l. The anthers were cultured in a petri dish and kept at 35°C in dark (8 days) and then transferred to 25°C and 16:8 h photoperiod (Dumas de Vaulx et al., 1981).

To find an optimal ratio of 2,4-D and Kinetin, the CP medium was supplemented with three concentrations of 2,4, D (Auxin) i.e., 0.25 (A_1), 0.50 (A_2), and 0.75 (A_3) mg/l along with four concentrations of Kinetin (cytokinin) i.e., 1.0(K_1), 2.0 (K_2), 3.0 (K_3) and 4.0 (K_4) mg/l. The anthers from buds were cultured in petri dishes at 35°C in the dark (8 days) and then transferred to 25°C and 16:8 h photoperiod (Dumas de Vaulx et al., 1981).

Best treatment combination factors from three experiments were noted individually between 30-60 days from the day of culture. These best results were combined in one experiment for testing androgenic response in all four genotypes under study using CP induction medium (Dumas de Vaulx et al., 1981).

RESULTS AND DISCUSSION

It is broadly acknowledged that anther culture advances the formation of microspore-derived embryos, in which the genotype has a remarkable impact on the rate of microspore deviation toward embryogenesis (Segui-Simarro and Nuez, 2008; Dunwell, 2010; Segui-Simarro, 2010; Irikova et al., 2011). Factors that turn on the gametophyte pathway instead of the sporophyte pathway during the incubation period of microspores are hot or cold pre-treatment, the composition of media, and the combination of plant growth regulators (PGRs).

The appearance and size of the buds along with microspore developmental stage and vitality are shown in Figure 1. In genotype V_1 (Asha) and V_4 (Indra), the bud size was in the range of 4.75–5.25 mm, while in genotype V_2 (Sympathy) and V_3 (Namelite), the bud size was in the range of 5.0-5.50 mm.Bud size having proper microspore development stage determines effective embryogenesis (Kim et al., 2004;Irikova et al., 2011). Our study confirmed the fact thatbud size ranging from 4.50-5.50 mm, with microspores from late uninucleate to early binucleate gavea higher embryonic response.These findings are corroborated byprevious studies conducted by Kim et al. (2004) and Supena et al. (2006a).The inoculation of healthy anthers on CP medium witha magnified view of embryos emerging from anthers and different developmental stages of embryos, are shown in figure 2.

The effect of cold pre-treatment (4°C) of buds at three different durations post anther culture and four respective heat shock durations for cultured anthers on CP medium at 35°C in dark +25°C-Cont. is given in table 1. The highest embryogenic response (39.3) was obtained in the combination $V_1C_2HS_3$ when buds of Asha (V_1) cold pre-treated at 4°C for 24 hours (C_2) and cultured anthers wereincubated at 35°C in dark for 10 days +25°C-Cont (HS_3) while lowest embryogenic response (0.0) was obtained in the combination $V_1xC_3xHS_1$ and was found at par with 13 other combinations i.e., $V_1C_1HS_4$,$V_1C_3HS_4$, $V_2C_1HS_1$, $V_2C_1HS_4$, $V_2C_3HS_1$, $V_2C_3HS_4$, $V_3C_1HS_4$, $V_3C_3HS_1$, $V_3C_3HS_2$, $V_3C_3HS_4$,

*e*ISSN1303-5150

$V_4C_1HS_4$, $V_4C_3HS_1$, and $V_4C_3HS_4$.The presentstudy revealed that cold pre-treatment of buds (4°C for 24 hours) C_2 and heat shock treatment after culturing of anthers (35°C in dark for 10 days +25°C-Cont light) HS_3 increases the number of embryos. These findings are in line with those of Sibi et al. (1979) and Dumas de Vaulx et al. (1981).

The effect of three different sucrose concentrationswithfour different concentrations of activated charcoal is shown in table 2. The highest embryogenic response (48.7) was obtained in combination with $V_1S_3AC_2$, where anthers of Asha (V_1) were excised and placed on the medium, which was supplemented with 4 % (S_3) sucrose along with 0.50 g/l (AC_2) of activated charcoal while lowest embryogenic response (0.00) was obtained in the combination $V_1S_1AC_1$ and found at par with 9 other combinations i.e., $V_2S_1AC_1$, $V_2S_1AC_3$, $V_2S_1AC_4$, $V_2S_2AC_1$, $V_2S_3AC_4$, $V_3S_1AC_1$, $V_3S_1AC_4$, $V_4S_1AC_1$, and $V_4S_1AC_4$.Activated charcoal and sucrose concentration were also previously shown to promote embryogenesis in anther culture (Johansson, 1983; Ciner and Tipirdamaz, 2002 and Dolcet-Sanjuan et al., 1997). In general, the role of activated charcoal is the absorption of inhibitory substances (phenolics, abscisic acid) released in medium during anther culture. In the present study, the highest numbers of embryos were obtained when medium was supplemented with 0.50 g/l of activated charcoal in combination with 4 % sucrose. However, Cinar and Tipirdamaz, 2002 obtained high embryogenic response using 0.25 % activated charcoal and 3% sucrose (Dumas de Vaulx et al. 1981).

The effect of auxin and cytokinin using 2,4-D and kinetin, respectively in different concentrationswere studied for effective embryogenesis. Three different concentrations of 2,4-D with four different kinetin concentrations are shown in table 3. The highest embryogenic response (43.0) was obtained in combination with $V_1A_2K_4$,where anther of hybrid Asha (V_1) were excised and placed on the medium, which was supplemented with 0.50 mg/l (A_2) 2,4-D and 4.0 mg/l (K_4) Kinetin, while lowest embryogenic response (00) was obtained in the combination $V_1A_3K_1$ and found at par with 9 other combinations i.e., $V_1A_3K_2$, $V_2A_1K_1$, $V_2A_3K_1$, $V_2A_3K_2$, $V_3A_3K_1$, $V_3A_3K_2$, $V_4A_1K_1$, $V_4A_1K_2$, and $V_4A_3K_1$. Growth regulators (auxins and cytokinin) play a vital role in microspore development and cell division in embryos (Chen, 1986). Grozeva et al., 2021and Hedari et al., 2017 used 0.30 mg/l of 2,4-D and 2.0 mg/l of kinetin,respectively, which gave a higher embryonic response (0.0%- 12.0%). Our study showed increased embryogenesis when combination of 2,4-D and kinetin was increased to 0.50 mg/l and 4.0 mg/l,respectively in the medium.

The best result from all previous experiments were combined and a new experiment was conducted in which the buds at the proper stage with maximum vitality were cold pre-treated at 4°C for 24 hours followed by culturing ofanthers fromcold pre-treated buds on medium which was supplemented with 4% sucrose, 0.50 gm/l activated charcoal, 0.50 mg/l 2,4-D and 4.0 mg/l kinetin with heat shock at 35°C for 10 days dark followed by transferring of plates to 25°C with 16:8 h photoperiod forever(Table 4). The experimental results showed highest number of embryos(34.7) in V_1 (Asha), while these werelowest in V_2 (Sympathy) (7.3). In terms of embryogenic induction frequency, the highest percentage (23.1%) was obtained in V_1 (Asha), while the lowest wasV_2 (Sympathy) (4.6%) (Figure 3).Previousresearchersworked with two or three factors on single or multiple genotypes (Chen 1986; Sibi et al., 1979 and Dolcet-Sanjuan et al., 1997), while we have extensively worked with six factors on four genotypes. The genotype is the most important and frequently a limiting factor in pepper androgenesis using anther culture (Morrison et al., 1986;Buyukalaca et al., 2004; Rodeva et al., 2004 and Koleva-Gudeva et al., 2007). It was confirmed in the present study, V_1 (Asha) was the most responsive genotype while V_2 (Sympathy) was the least.

Conclusion

In sweet pepper androgenesis, anther culture is the most effective system as it is widely used by various researchers. One of the

crucial factors for embryogenesis is the responsiveness of genotype, so it is advised to take moregenotypes for experimentation. Our results suggest that a proper combination of factors can bring overall increase in the embryogenicresponse in sweet pepper. Further research is required for more embryogenic responses in sweet pepper.

ACKNOWLEDGEMENT
The authors acknowledge Department of Biotechnology in ACSEN HYVEG Pvt. Ltd. Kullu, H.P. for providing facilities to carry out this work and GLA University, Mathura U.P. for providing guidance whenever required.

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Table 1. Effect of cold pretreatment and heat shock on androgenic response in sweet pepper

Variety	Cold pre-treatment at 4°C (Hours)	Heat Shock				Mean
		HS_1 (35°C for 6 days dark + 25°C cont…)	HS_2 (35°C for 8 days dark + 25°C cont…)	HS_3 (35°C for 10 days dark + 25°C cont…)	HS_4 (35°C for 12 days dark + 25°C cont…	
V1 (Asha)	C_1 (0)	4.7 ± 0.3^{lmn}	7.7 ± 0.9^{ij}	16.3 ± 0.3^{d}	1.0 ± 0.0^{rs}	7.4 ± 0.2^{d}
	C_2 (24)	10.3 ± 0.3^{fg}	16.0 ± 0.6^{d}	39.3 ± 0.3^{a}	7.0 ± 0.6^{jk}	18.2 ± 0.2^{a}
	C_3 (48)	0.0 ± 0.0^{s}	2.7 ± 0.7^{opq}	10.7 ± 0.3^{fg}	1.3 ± 07^{qrs}	3.7 ± 0.3^{g}
V2 (Sympathy)	C_1 (0)	0.0 ± 0.0^{s}	$27.\pm0.3^{opq}$	4.7 ± 0.3^{lmn}	1.0 ± 0.0^{rs}	2.1 ± 0.2^{h}
	C_2 (24)	3.3 ± 0.7^{nop}	4.3 ± 0.3^{mno}	7.0 ± 0.6^{jk}	3.3 ± 0.7^{nop}	4.5 ± 0.1^{f}
	C_3 (48)	0.0 ± 0.0^{s}	3.3 ± 0.7^{nop}	4.0 ± 1.2^{mnop}	0.0 ± 0.0^{s}	1.8 ± 0.3^{h}
V3 (Namelite)	C_1 (0)	2.3 ± 0.3^{pqr}	7.3 ± 0.3^{ijk}	14.3 ± 0.7^{e}	0.0 ± 0.0^{s}	6.0 ± 0.0^{e}
	C_2 (24)	6.0 ± 0.6^{kl}	11.3 ± 0.3^{f}	20.3 ± 0.7^{c}	2.7 ± 0.3^{opq}	10.1 ± 0.3^{c}
	C_3 (48)	0.0 ± 0.0^{s}	$1.0.\pm0.6^{rs}$	3.0 ± 0.6^{nop}	0.0 ± 0.0^{s}	1.0 ± 0.3^{i}
V4 (Indra)	C_1 (0)	4.0 ± 0.6^{mnop}	5.3 ± 0.7^{lm}	9.7 ± 0.7^{gh}	1.0 ± 0.6^{rs}	5.0 ± 0.0^{f}
	C_2 (24)	6.0 ± 0.6^{kl}	8.7 ± 0.7^{hi}	28.0 ± 0.6^{b}	5.3 ± 0.3^{lm}	12.0 ± 0.1^{b}
	C_3 (48)	0.0 ± 0.0^{s}	3.3 ± 0.7^{nop}	6.0 ± 0.6^{kl}	0.3 ± 0.3^{s}	2.4 ± 0.2^{h}
	Mean	3.1 ± 0.1^{c}	6.1 ± 0.1^{b}	13.6 ± 0.1^{a}	1.9 ± 0.0^{d}	

Factors	C.D.(0.01)	±SE(d)	±SE(m)
Variety (V)	0.529	0.266	0.188
Cold pre-treatment (C)	0.458	0.231	0.163
Heat shock (HS)	0.529	0.266	0.188
Interaction (V×C)	0.916	0.461	0.326
Interaction(V×HS)	1.058	0.533	0.377
Interaction(C×HS)	0.916	0.461	0.326

Mean values of interaction (V×C×HS) and overall mean of each variety (in row) and heat shock (in column) followed by the samelower case letters were not significantly different at P ≤ 0.05 according to Duncan's multiple range test

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Table 2. Effect of sucrose and activated charcoal on androgenic response in sweet pepper

Variety	Sucrose (%)	Activated Charcoal (g/l)				
		AC_1 (0.25)	AC_2 (0.50)	AC_3 (0.75)	AC_4 (1.0)	Mean
V1 (Asha)	S_1 (2)	0.0 ± 0.0^{u}	4.7 ± 0.3^{nop}	4.0 ± 0.6^{opq}	2.7 ± 0.3^{qrs}	2.8 ± 0.2^{h}
	S_2 (3)	7.7 ± 0.3^{kl}	22.0 ± 0.6^{d}	8.0 ± 0.6^{k}	6.0 ± 0.6^{lmn}	10.9 ± 0.2^{d}
	S_3 (4)	12.0 ± 0.6^{hi}	48.7 ± 0.7^{a}	23.3 ± 0.7^{d}	15.0 ± 1.0^{g}	24.8 ± 0.4^{a}
V2 (Sympathy)	S_1 (2)	0.0 ± 0.0^{u}	2.0 ± 0.6^{rst}	0.3 ± 0.3^{tu}	0.0 ± 0.0^{u}	0.6 ± 0.1^{i}
	S_2 (3)	0.0 ± 0.0^{u}	4.3 ± 0.9^{nop}	4.0 ± 0.6^{opq}	2.0 ± 0.6^{rst}	2.6 ± 0.5^{h}
	S_3 (4)	10.0 ± 0.6^{j}	10.7 ± 0.9^{ij}	5.3 ± 0.3^{mno}	0.0 ± 0.0^{u}	6.5 ± 0.2^{f}
V3 (Namelite)	S_1 (2)	0.7 ± 0.3^{tu}	5.3 ± 0.3^{mno}	3.0 ± 0.6^{pqr}	1.0 ± 0.6^{stu}	2.5 ± 0.3^{h}
	S_2 (3)	8.0 ± 0.6^{k}	19.0 ± 0.6^{e}	7.0 ± 1.0^{klm}	3.0 ± 0.6^{pqr}	9.3 ± 0.4^{e}
	S_3 (4)	12.3 ± 0.9^{hi}	32.0 ± 0.6^{c}	12.7 ± 0.7^{h}	9.7 ± 0.7^{j}	16.7 ± 0.5^{b}
V4 (Indra)	S_1 (2)	0.0 ± 0.0^{u}	17.0 ± 0.6^{f}	2.0 ± 0.0^{rst}	0.0 ± 0.0^{u}	4.8 ± 0.1^{g}
	S_2 (3)	6.0 ± 0.6^{lmn}	22.0 ± 0.6^{d}	6.0 ± 0.6^{lmn}	5.0 ± 0.6^{no}	9.8 ± 0.1^{e}
	S_3 (4)	7.0 ± 0.6^{klm}	37.3 ± 0.3^{b}	12.3 ± 0.9^{hi}	3.0 ± 0.6^{pqr}	15.0 ± 0.4^{c}
	Mean	5.3 ± 0.21^{c}	18.8 ± 0.2^{a}	7.3 ± 0.0^{b}	3.9 ± 0.0^{d}	

Factors	C.D.(0.01)	±SE(d)	±SE(m)
Variety (V)	0.409	0.206	0.146
Sucrose (S)	0.354	0.178	0.126
Activated Charcoal (AC)	0.409	0.206	0.146
Interaction (V×S)	0.708	0.357	0.252
Interaction (V×AC)	0.409	0.206	0.146
Interaction (S×AC)	0.818	0.412	0.291
Interaction (V×S×AC)	0.708	0.357	0.252

Mean values of interaction (V×S×AC) and overall mean of each variety (in row) and activated charcoal (in column) followed by the same lower case letters were not significantly different at P ≤ 0.05 according to Duncan's multiple range test

Table 3. Effect of auxin and cytokinin on androgenic response in sweet pepper

Variety	2,4-D(mg/l)	Kinetin(mg/l)				
		K_1 (1.0)	K_2 (2.0)	K_3 (3.0)	K_4 (4.0)	Mean
V1 (Asha)	A_1 (0.25)	2.3 ± 0.3^{stu}	5.0 ± 0.6^{mnop}	18.0 ± 0.6^{fg}	28.0 ± 0.6^{d}	13.3 ± 0.3^{c}
	A_2 (0.50)	5.7 ± 0.3^{mn}	10.3 ± 0.7^{jk}	24.0 ± 0.6^{e}	43.0 ± 0.6^{a}	20.8 ± 0.3^{a}
	A_3 (0.75)	0.0 ± 0.0^{v}	0.0 ± 0.0^{v}	2.0 ± 0.6^{tu}	6.0 ± 0.6^{m}	2.0 ± 0.1^{h}
V2 (Sympathy)	A_1 (0.25)	0.0 ± 0.0^{v}	2.7 ± 0.3^{rst}	4.3 ± 0.3^{nopq}	6.0 ± 0.6^{m}	3.3 ± 0.21^{g}
	A_2 (0.50)	5.3 ± 0.3^{mno}	5.3 ± 0.3^{mno}	6.3 ± 0.3^{m}	11.7 ± 0.3^{ij}	7.2 ± 0.2^{d}
	A_3 (0.75)	0.0 ± 0.0^{v}	0.0 ± 0.0^{v}	2.0 ± 0.6^{tu}	3.0 ± 0.6^{qrst}	1.3 ± 0.3^{i}
V3 (Namelite)	A_1 (0.25)	2.0 ± 0.6^{tu}	4.0 ± 0.6^{opqr}	6.0 ± 0.6^{m}	13.0 ± 0.6^{i}	6.3 ± 0.3^{e}

	A_2 (0.50)	5.3±0.3^{mno}	8.7±0.3^{l}	19.3±0.7^{f}	32.3±0.3^{c}	16.4±0.1^{b}
	A_3 (0.75)	0.0±0.0^{v}	1.0±0.6^{uv}	3.0±0.6^{qrst}	5.7±0.3^{mn}	2.4±0.2^{h}
	A_1 (0.25)	0.0±0.0^{v}	1.0±0.6^{uv}	3.3±0.3^{qrst}	14.7±0.9^{h}	4.8±0.0^{f}
V4 (Indra)	A_2 (0.50)	4.0±0.6^{opqr}	10.7±0.9^{j}	16.7±0.9^{g}	34.0±0.6^{b}	16.3±0.3^{b}
	A_3 (0.75)	0.3±0.3^{v}	3.7±0.3^{pqrs}	5.0±0.6^{mnop}	9.0±0.6^{kl}	4.5±0.3^{f}
	Mean	2.1±0.1^{d}	4.4±0.0^{c}	9.2±0.1^{b}	17.2±0.2^{a}	

Factors	C.D.(0.01)	±SE(d)	±SE(m)
Variety (V)	0.403	0.203	0.144
2,4-D (A)	0.349	0.352	0.249
Kinetin (Kn)	0.403	0.203	0.144
Interaction (V×A)	0.699	0.352	0.249
Interaction (V×Kn)	0.807	0.406	0.287
Interaction (A×Kn)	0.699	0.352	0.249
Interaction (V×A×Kn)	1.397	0.704	0.498

Mean values of interaction (V×A×Kn) and overall mean of each variety (in row) and kinetin (in column) followed by the same lower case letters were not significantly different at P ≤ 0.05 according to Duncan's multiple range test

Table 4. Androgenic response of different genotypes of using best treatment combinations*

Genotype	No of explant culture	No. of embryos/150 anthers#
V1 (Asha)	150	34.7±0.9 ^{a}
V2 (Sympathy)	150	7.3±0.9 ^{d}
V3 (Namelite)	150	22.3±0.3 ^{c}
V4 (Indra)	150	27.3±0.9 ^{b}

#mean values are significantly different at P ≤ 0.05 according to Duncan's multiple range test
*Best results of experiments 1, 2, 3 (Treatment of bud at 4oC for 24 hours followed by placing of excised anther on media which is supplemented with 4% sucrose, 0.50 g of activated charcoal, 0.50 mg/l of 2,4-D, 4.0 mg/l of kinetin and giving heat shock at 35oC for 10 days followed by transferring of plates to 25°C and 16:8 h photoperiod forever)

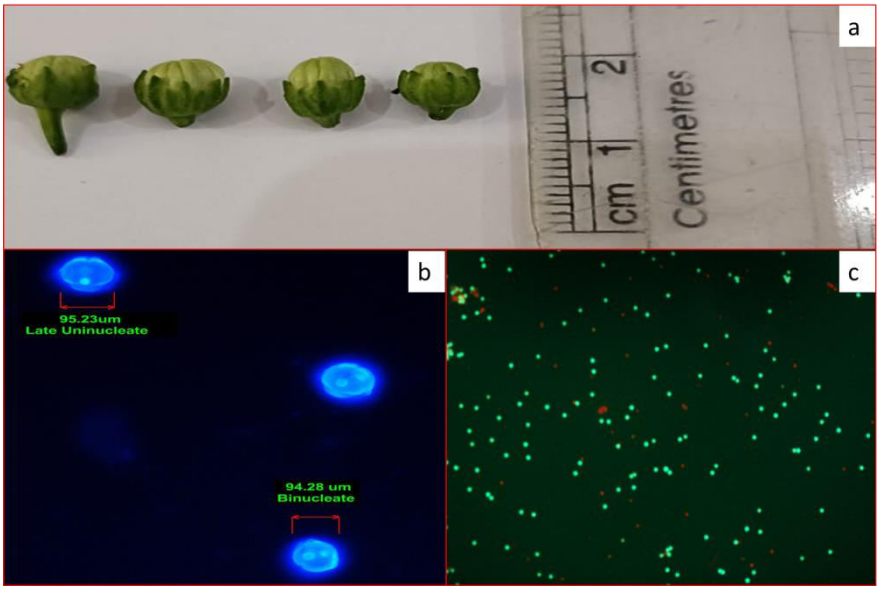

Figure 1: Different sizes of buds (a), microspore stage for the anther culture; late uninucleate and binucleate microspore determined by DAPI (b), and Vitality of microspore by FDA/PI staining. (Viable cells –green & dead cells-Red) (c)

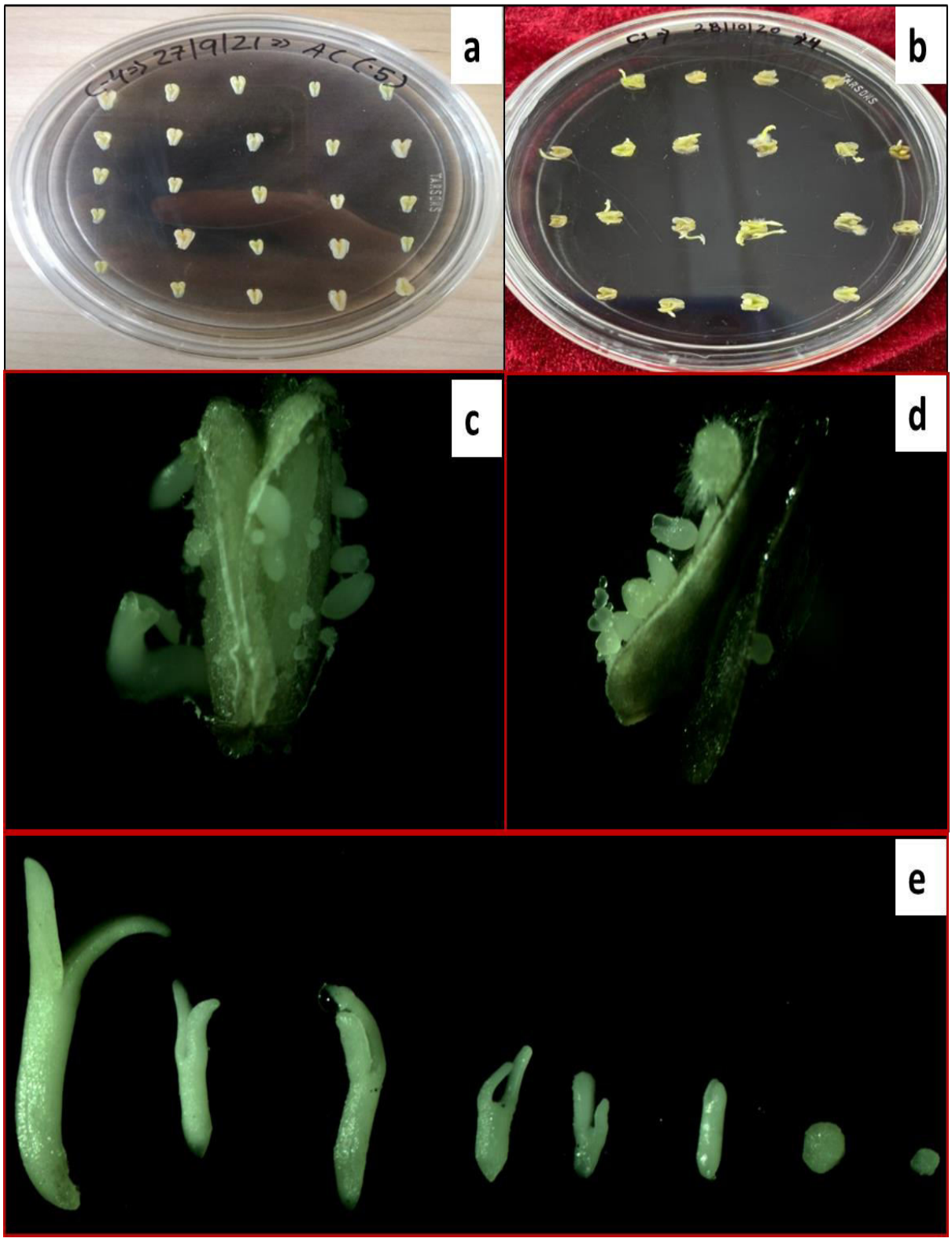

Figure 2: Anthers inoculation on media (a), emerging embryos from cultured anthers (b), magnified view of the emerging embryo from anther wall (c) (d), and different developmental stages of embryos i.e., globular to cotyledonary (e).

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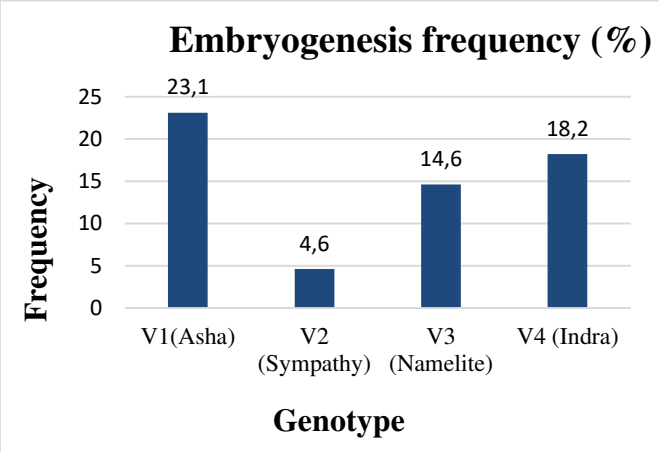

Figure 3: Embryogenesis frequency of different genotypes by using best treatment combinations*
*Best results of experiments 1, 2, 3 (Treatment of bud at 4°C for 24 hours followed by placing of excised anther on media which is supplemented with 4% sucrose, 0.50 g of activated charcoal, 0.50 mg/l of 2,4-D, 4.0 mg/l of kinetin and giving heat shock at 35°C for 10 days followed by transferring of plates to 25°C and 16:8 h photoperiod forever)

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*e*ISSN1303-5150