RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
56 (5) 2079-2089, 2024
http://doi.org/10.54910/sabrao2024.56.5.30
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

# FLORAL BUD SIZE AND CULTURE CONDITIONS' EFFECT ON EMBRYOGENESIS IN ANTHER-DERIVED CALLI OF CUCUMBER

**F. KHADIJA[1,2], B. FATIMA[1*], M. USMAN[1], and M.S. KHAN[3]**

[1]Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan
[2]Mango Research Station, Shujabad, Pakistan
[3]Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan
*Corresponding author's email: dr.fatimausman@uaf.edu.pk
Email addresses of co-authors: fkdurani@yahoo.com, m.usman@uaf.edu.pk, sarwarkhan_40@hotmail.com

## SUMMARY

Cucumber (*Cucumis sativus* L.) is a genetically diverse group of vegetables with various cultivars having distinct traits. This study optimized somatic embryogenesis in anther-derived calli of selected commercial cucumber cultivars (Local Khera, Champion, and CP 001). Therefore, the experiment investigated the impact of 2,4-D and benzyl 6-aminopurine (BAP) treatments on embryo formation. The anthers collected from different-sized floral buds sustained culturing in various concentrations of 2,4-D and BAP (0, 0.5, 1, 1.5, 2, 3, and 4 mg $L^{-1}$). It was evident that calli induction in cucumber cultivars received significant stimuli from 2,4-D and BAP concentrations and dark culture conditions during calli culture. The maximum calli induction (51%) was prominent in anthers of cv. CP 001 at a higher level of 2,4-D in small-sized floral buds. However, the anthers of cucumber cultivar Local Khera (59.72%) performed better for calli induction than Champion (57.14%) and CP 001 (51.43%). The highest embryogenesis appeared in anther-derived calli of cultivar Local Khera (12%) under light culture conditions in tiny flower buds. Meanwhile, maximum (8%) embryo formation observed at a higher level of 2,4-D (4 mg $L^{-1}$) resulted in cultivars Champion and CP 001 under dark conditions. In conclusion, from the tested treatments, applying the highest level of 2,4-D and BAP at 4 mg $L^{-1}$ was more effective than other treatments, including the control. However, more calli induction was noteworthy under dark culture conditions, and maximum embryo formation occurred under light culture conditions.

**Keywords:** Cucumber (*C. sativus* L.), cultivars, anthers, dark, light, calli, embryo

Communicating Editor: Prof. Z.A. Soomro

Manuscript received: November 29, 2023; Accepted: May 19, 2024


**Citation:** Khadija F, Fatima B, Usman M, Khan MS (2024). Floral bud size and culture conditions' effect on embryogenesis in anther-derived calli of cucumber. *SABRAO J. Breed. Genet.* 56(5): 2079-2089. http://doi.org/10.54910/sabrao2024.56.5.30.

**Key findings:** Applying the highest level of 2,4-D and BAP at 4.0 mg $L^{-1}$ was the most effective for calli production and embryogenesis in cucumber (*C. sativus* L.). However, more calli induction was prominent under dark culture conditions, while maximum embryo formation occurred under light culture conditions.

## INTRODUCTION

The cucumber is an economic summer vegetable mainly used as a salad crop. During 2021, cucumbers' approximate total world production was 93.5 million tons (FAO, 2023). Cucumber growing occupied a total area of 3,573 hectares with a total yield of 112,231 tons in Pakistan during 2022–2023 (GOP, 2024). China is the leading producer worldwide in area and production of cucumbers (Sreenayana and Nakkeeran, 2019). China has a share of 62.7% of the sub-total area and 63.59% of the world's annual production. Cucumber production in Pakistan is less than that of the leading countries globally.

Embryogenesis and organogenesis are two applicable techniques used in vitro for developing plants from calli, embryoids, or tissues. The first report on cucumber anther culture came out in 1982 without plantlet regeneration (Lazarte and Sasser, 1982). The microspore and anthers serve in developing haploid plants utilized to produce homozygous diploids in plant improvement programs (Gałązka *et al*., 2013; Abo-Shama and Atwa, 2019; Ali *et al*., 2023) and as a source of genetic variations (Wernsman, 1992).

Many factors affect the development of haploids through anther culture, including the condition of donor plant growth, pre-treatment of anthers or buds, genotype, incubation conditions, and microspore development stage (Hamidvand *et al*., 2013; Usman *et al*., 2015; Domblides *et al*., 2019; Bokhari *et al*., 2023). Previous reports stated that seasonal variations influenced anther response in different potato cultivars (Abo-Shama and Atwa, 2019). The percentage of anthers that produced embryos stands between 15%–20% in the 9th and 10th month of the year and 1% to 3% in the 2nd and 5th month of the year (Kaur *et al*., 2018; Abo-Shama and Atwa, 2019) and in cucumber (Usman *et al*., 2011, 2015; Abdollahi *et al*., 2016).

Anther culture is also essential in developing genetically improved variants and transferring the interspecific gene (Wernsman, 1992). Therefore, anther/microspore culture is beneficial for genetic improvement (Malepszy, 1988). Earlier reports had androgenesis in different members of Cucurbitaceae, such as watermelon (Jaskani *et al*., 2004) and bitter gourd (Usman *et al*., 2015). The production of haploid plants in cucumber using anther culture would direct cucumber breeders to develop new lines. The latest study aimed to evaluate the effect of genotype, explant age, plant growth regulators, and light and dark culture conditions on embryogenesis in anther-derived calli and optimize androgenesis for haploidization.

## MATERIALS AND METHODS

The presented research commenced in the Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan.

### Explant source and sterilization procedures

The floral bud collections came from the vegetable research area, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, and Ayyub Agricultural Research Institute, Faisalabad. However, the small- (11–13 mm) and medium-sized (13–15 mm) floral buds became samples in the study (Figure 1A). Anthers collected from unopened buds and pollens underwent observation after staining under the microscope for the proper stage of development. The buds containing anthers under the uni-nucleate/bi-nucleate stage were opted as a source of anthers for in vitro culture. Buds received surface disinfection with 70% Ethanol (v/v) + 1–2 drops of Tween-20 detergent for 2–3 min, followed by 2–3

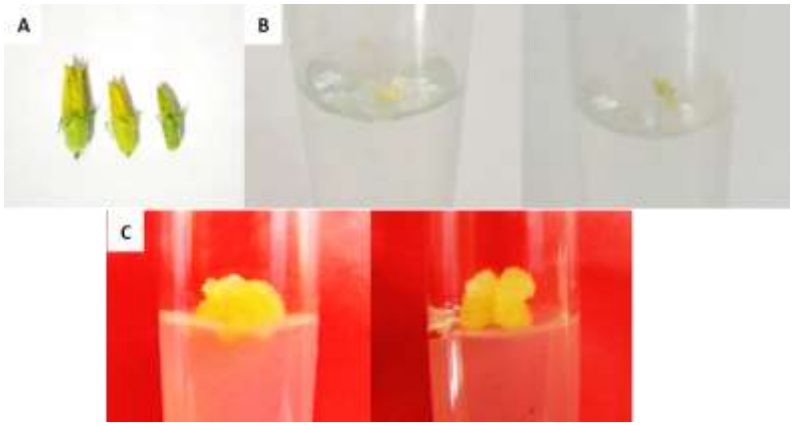


**Figure 1.** Callogenesis and embryogenic responses in anthers of cucumber cultivars cultured on MS medium supplemented with 2,4-D. Figures show A) Floral bud selection B) callus induction and C) formation of pro-embryogenic masses in anther derived proliferating calli.

rinses with sterilized water. Then, the floral buds proceeded to dip in 5% sodium hypochlorite (v/v) for 5–8 min, followed by 3–5 rinses with sterilized water before culturing in the media. After petal removal aseptically, the anthers were carefully excised with forceps and placed on the following media:

***Media formulation***

Anther culture on Murashige and Skoog (MS) (1962) medium had supplements of different auxin and cytokinins for calli induction, and calli induced by the same treatments continued growth on the MS media for embryogenesis (Table 1).

***Culture conditions***

After inoculating, the cultures proceeded in the growth room with a temperature of 25 °C±2 °C facilitated with 1000 lux of light intensity.

**Experimental layout**

The experiment proceeded in a completely randomized design (CRD), with three replications per treatment. Ten tubes for culturing consisted of each replication. The collected data was analysed, and the means were compared for the Least Significant Difference (LSD) test (Steel *et al*., 1997).

**RESULTS**

**Calli induction percentage**

***A. 2,4-dichlorophenoxyacetic acid (2,4-D)***

All the treatments of 2,4-D (0.5, 1, 1.5, 2, 3, and 4 mg $L^{-1}$) posed significant effects ($P \leq 0.05$) on the calli induction percentage (%) under light and dark culture conditions of the anther culture with the small- to medium-sized flower buds among three selected cucumber cultivars (Champion, CP 001, and Local Khera) (Table 2). During light culture conditions, the lowest calli induction percentage (28.33%) was evident in medium-sized buds, followed by small-sized buds (30.38%) for the control (without 2,4-D) in the Local Khera cultivar. However, maximum calli induction (51.43%) was prominent at a higher level at 4 mg $L^{-1}$ 2,4-D in small-sized flower buds of the cultivar CP 001 (Figure 1B). A similar trend resulted in CP 001 under light culture conditions of medium-sized flower buds. Under dark culture conditions, it was apparent that the Champion cultivar showed a maximum calli induction percentage (48.89%) in small-sized flower buds when cultured at the highest concentration of 4 mg $L^{-1}$ 2,4-D followed by 3 mg $L^{-1}$ in small-sized buds (48.61%). A similar trend occurred in medium-sized flower buds.

**Table 1.** MS media modified with different concentrations of plant growth regulators (PGRs).

| Treatments | Plant growth regulators (mg $L^{-1}$) | | | |
|---|---|---|---|---|
| | Light Conditions (16/8 hr photoperiod) | | Dark conditions (24 hr darkness) | |
| | 2,4-D | BAP | 2,4-D | BAP |
| $T_0$ | Control | Control | Control | Control |
| $T_I$ | 0.5 | 0.5 | 0.5 | 0.5 |
| $T_2$ | 1.0 | 1.0 | 1.0 | 1.0 |
| $T_3$ | 1.5 | 1.5 | 1.5 | 1.5 |
| $T_4$ | 2.0 | 2.0 | 2.0 | 2.0 |
| $T_5$ | 3.0 | 3.0 | 3.0 | 3.0 |
| $T_6$ | 4.0 | 4.0 | 4.0 | 4.0 |

**Table 2.** Effect of 2,4-D on calli induction (%) from anthers with different bud sizes from different cultivars of cucumber under light and dark culture conditions.

| MS+2,4-D (mg $L^{-1}$) | Light culture conditions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Local Khera | Champion | CP 001 | Means | Local Khera | Champion | CP 001 | Means |
| | Small flower size bud (11–13 mm) | | | | Medium flower size bud (13–15) mm | | | |
| Control | 30.38 j | 31.43 ij | 31.43 ij | 31.08 E | 28.33 | 30.00 | 28.33 | 28.89 E |
| 0.5 | 40.11 fg | 37.14 gh | 34.29 hi | 37.18 D | 34.17 | 35.33 | 34.17 | 34.56 D |
| 1.0 | 40.95 ef | 37.09 gh | 34.29 hi | 37.44 D | 35.83 | 35.83 | 35.00 | 35.55 D |
| 1.5 | 40.86 ef | 40.00 fg | 36.19 h | 39.02 D | 37.50 | 36.33 | 37.67 | 37.17 C |
| 2.0 | 45.71 cd | 41.52 ef | 40.00 fg | 42.41 C | 38.50 | 37.50 | 37.50 | 37.83 BC |
| 3.0 | 43.81 cde | 42.86 def | 46.67 bc | 44.45B | 40.00 | 39.00 | 38.33 | 39.11 B |
| 4.0 | 45.71 cd | 49.52 ab | 51.43 a | 48.89 A | 42.50 | 42.00 | 43.00 | 42.50 A |
| Means | 41.08 A | 39.94 AB | 39.19 B | | 36.69 | 36.57 | 36.29 | |
| LSD *(P ≤ 0.05)* | | | | | | | | |
| Treatments (T) | | 1.99 | | | 1.30 | | | |
| Cultivars (C) | | 1.31 | | | NS | | | |
| T × C | | 3.46 | | | NS | | | |
| MS+2,4-D (mg $L^{-1}$) | Dark culture conditions | | | | | | | |
| | Local Khera | Champion | CP 001 | Means | Local Khera | Champion | CP 001 | Means |
| | Small flower size bud (11–13 mm) | | | | Medium flower size bud (13–15) mm | | | |
| Control | 31.17 j | 38.89 gh | 30.00 j | 33.35 F | 29.00 m | 37.50 j | 27.78 n | 31.43 G |
| 0.5 | 38.89 gh | 40.28 fgh | 34.72 i | 37.96 E | 33.33 l | 43.06 g | 36.11 k | 37.50 F |
| 1.0 | 40.28 fgh | 43.06 def | 37.50 hi | 40.28 D | 38.89 i | 44.44 f | 38.89 i | 40.74 E |
| 1.5 | 42.67 ef | 45.83 bcd | 37.50 hi | 42.00 C | 41.67 h | 51.39 d | 38.89 i | 43.98 D |
| 2.0 | 45.50 cd | 48.61 ab | 40.89 e-g | 44.99 B | 43.06 g | 54.17 c | 41.67 h | 46.30 C |
| 3.0 | 47.00 abc | 48.61 ab | 43.61 de | 46.41 AB | 43.06 g | 55.56 b | 43.06 g | 47.23 B |
| 4.0 | 48.15 abc | 48.89 a | 46.44 abc | 47.82 A | 44.44 f | 56.94 a | 45.83 e | 49.07 A |
| Means | 41.95 B | 44.88 A | 38.67 C | | 39.06 B | 49.01 A | 38.89 B | |
| LSD *(P ≤ 0.05)* | | | | | | | | |
| Treatments (T) | | 1.62 | | | 0.60 | | | |
| Cultivars (C) | | 1.06 | | | 0.39 | | | |
| T × C | | 2.80 | | | 1.03 | | | |

Where T, treatments; C, cultivar; any two means within a column followed by different letters are significantly different, while same letters within a column means non-significant (NS) difference.

***B. 6-Benzylaminopurine (BAP)***

Outcomes revealed that the Local Khera showed maximum calli induction percentage (52.50%) in medium-sized buds when cultured at the highest concentration of 4.0 mg $L^{-1}$ benzylaminopurine (BAP) (Table 3). The lowest calli induction percentage (30%) appeared in medium-sized buds, followed by small-sized buds (31.43%) of the control under light

**Table 3.** Effect of BAP on calli induction (%) from anthers with different bud sizes from different cultivars of cucumber under light and dark culture conditions.

| MS+ BAP (mg $L^{-1}$) | Light culture conditions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Local Khera | Champion | CP 001 | Means | Local Khera | Champion | CP 001 | Means |
| | Small flower size bud (11–13 mm) | | | | Medium flower size bud (13–15) mm | | | |
| Control | 33.33 l | 31.43 m | 31.43 m | 32.06 G | 32.50 i | 30.00 j | 32.50 i | 31.67 F |
| 0.5 | 37.14 i | 34.29 k | 36.19 j | 35.87 F | 34.00 hi | 34.17 h | 35.83 g | 34.67 E |
| 1.0 | 39.05 h | 37.14 i | 37.14 i | 37.78 E | 37.50 f | 36.67 fg | 37.50 f | 37.23 D |
| 1.5 | 40.00 g | 42.86 f | 42.86 f | 41.91 D | 42.50 d | 39.17 e | 39.17 e | 40.28 C |
| 2.0 | 42.86 f | 42.86 f | 43.81 de | 43.18 C | 43.17 d | 40.00 e | 40.00 e | 41.06 C |
| 3.0 | 45.71 c | 43.44 e | 44.00 d | 44.38 B | 49.17 b | 42.00 d | 43.00 d | 44.73 B |
| 4.0 | 47.62 b | 48.57 a | 46.00 c | 47.40 A | 52.50 a | 45.00 c | 45.00 c | 47.50 A |
| Means | 40.82 A | 40.08 B | 40.20 B | | 41.62 A | 38.14 C | 39.00 B | |
| LSD *(P ≤ 0.05)* | | | | | | | | |
| Treatments (T) | | 0.24 | | | 0.92 | | | |
| Cultivars (C) | | 0.16 | | | 0.60 | | | |
| T × C | | 0.41 | | | 1.59 | | | |
| MS+ BAP (mg $L^{-1}$) | Dark culture conditions | | | | | | | |
| | Local Khera | Champion | CP 001 | Means | Local Khera | Champion | CP 001 | Means |
| | Small flower size bud (11–13 mm) | | | | Medium flower size bud (13–15) mm | | | |
| Control | 30.48i | 31.43 i | 31.43 i | 31.11 E | 30.00 | 32.5 | 28.33 | 30.28 E |
| 0.5 | 40.00 f | 37.14 g | 34.29 h | 37.14 D | 34.17 | 35.00 | 34.17 | 34.45 D |
| 1.0 | 40.95 ef | 37.14 g | 36.19 gh | 38.09 D | 35.83 | 35.83 | 35.00 | 35.55 D |
| 1.5 | 42.86 de | 42.00 def | 37.14 g | 40.67 C | 37.50 | 37.50 | 36.67 | 37.22 C |
| 2.0 | 43.81 cd | 40.00 f | 40.00 f | 41.27 C | 37.50 | 37.56 | 37.50 | 37.52 BC |
| 3.0 | 45.71 bc | 42.86 de | 46.67 b | 45.08 B | 40.00 | 38.00 | 42.50 | 38.78 B |
| 4.0 | 45.71 bc | 49.52 a | 47.00 b | 47.41 A | 42.50 | 40.00 | 43.00 | 41.83 A |
| Means | 41.36A | 40.01 B | 38.96 C | | 36.79 | 36.63 | 36.14 | |
| LSD *(P ≤ 0.05)* | | | | | | | | |
| Treatments (T) | | 1.21 | | | 1.53 | | | |
| Cultivars (C) | | 0.79 | | | NS | | | |
| T × C | | 2.09 | | | NS | | | |

Where T, treatments; C, cultivar; any two means within a column followed by different letters are significantly different, while same letters within a column means non-significant (NS) difference.

culture conditions. Meanwhile, the cultivar Champion gave the maximum calli induction percentage (48.57%) in small-sized flower buds when cultured at the higher concentration of 4 mg $L^{-1}$ BAP, followed by the Local Khera (47.62%) and CP 001 (46%) at the same level of BAP under light culture conditions. Under dark culture conditions, the cultivar Champion provided the maximum calli induction percentage (49.52%) in small-sized floral buds when cultured at the highest concentration of 4 mg $L^{-1}$ BAP, followed by CP 001 (47%) and Local Khera (45.71%). The lowest calli induction percentage in medium-sized flower buds was 28.33% at the control under dark culture conditions. Comparing means of the 4 mg $L^{-1}$ BAP treatment showed a maximum calli induction percentage (41.83%) versus the control (30.28%) in medium-sized floral buds.

**Embryogenesis (%) under light culture conditions**

***A. 2,4-dichlorophenoxyacetic acid (2,4-D)***

The results showed that embryogenesis (%) significantly increased when raising the level of 2,4-D (3 and 4 mg $L^{-1}$) from anthers of small floral buds (11–13 mm) (Table 4). The data indicated that maximum embryo formation (12%) was evident in cultivar Local Khera, followed by CP 001 (9%) at a higher level of 4 mg $L^{-1}$ 2,4-D (Figure 1C). However, minimum embryogenesis (2.33%) resulted in CP 001,

**Table 4.** Embryogenesis (%) in anther derived calli on MS + 2,4-D and BAP from small and medium size flower buds under light culture conditions.

| MS medium+ 2,4-D (mg L-1) | Embryogenesis (%) under light culture conditions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Local Khera | Champion | CP 001 | Means | Local Khera | Champion | CP 001 | Means |
| | Small flower size bud (11–13 mm) | | | | Medium flower size bud (13–15) mm | | | |
| Control | 4.17 hi | 3.00 ij | 2.33 j | 3.17 E | 3.00 g | 5.00 ef | 3.00 g | 3.67 D |
| 0.5 | 6.00 fg | 4.33 hi | 4.67 gh | 5.00 D | 4.00 fg | 5.00 ef | 3.00 g | 4.00 D |
| 1.0 | 6.00 fg | 5.67 fgh | 5.67 fgh | 5.78CD | 6.00 de | 6.00 de | 6.00 de | 5.67 C |
| 1.5 | 6.00 fg | 6.00 fg | 6.33 ef | 6.11C | 8.00 bc | 7.67 bc | 6.00 de | 7.22 B |
| 2.0 | 9.00 bc | 6.33 ef | 7.00 def | 7.44B | 6.67 cd | 8.00 bc | 7.00 cd | 7.22 B |
| 3.0 | 10.00 b | 6.67 def | 8.00 cd | 8.22B | 8.00 bc | 8.00 bc | 7.00 cd | 7.67 B |
| 4.0 | 12.00 a | 7.67 cde | 9.00 bc | 9.56 A | 10.00 a | 9.00 ab | 10.00 a | 9.67 A |
| Means | 7.59 A | 5.67 B | 6.15 B | | 6.52 A | 6.95A | 5.86 B | |
| LSD (*P* ≤ *0.05*) | | | | | | | | |
| Treatments (T) | | 0.9 | | | 0.9 | | | |
| Cultivars (C) | | 0.59 | | | 0.59 | | | |
| T × C | | 1.55 | | | 1.55 | | | |
| MS+ BAP (mg L-1) | Local Khera | Champion | CP 001 | Means | Local Khera | Champion | CP 001 | Means |
| | Small flower size bud (11–13 mm) | | | | Medium flower size bud (13–15) mm | | | |
| Control | 2.67 fg | 2.00 g | 4.00 ef | 2.89 E | 1.67 ij | 1.33 j | 2.33 hij | 1.78D |
| 0.5 | 4.00 ef | 2.67 fg | 5.00 de | 3.89 D | 3.00 ghi | 1.67 ij | 4.00 efg | 2.89 C |
| 1.0 | 6.00 cd | 3.00 fg | 7.00 bc | 5.33 C | 4.67 def | 2.00 ij | 5.33 bcde | 4.00 B |
| 1.5 | 8.00 ab | 4.00 ef | 8.00 ab | 6.67 B | 6.00 abcd | 3.00 ghi | 3.00 ghi | 5.00 A |
| 2.0 | 7.00 bc | 5.00 de | 8.00 ab | 6.67 B | 6.33 abc | 3.67 fgh | 5.67 abcd | 5.22 A |
| 3.0 | 8.00 ab | 5.00 de | 9.00 a | 7.33 AB | 6.67 ab | 4.67 def | 6.00 abcd | 5.78 A |
| 4.0 | 8.00 ab | 6.00 cd | 9.00 a | 7.67A | 7.00 a | 5.00 cdef | 5.33 bcde | 5.78 A |
| Means | 6.24 B | 3.95 C | 7.14 A | | 5.05 A | 3.05 B | 4.95 A | |
| LSD (*P* ≤ *0.05*) | | | | | | | | |
| Treatments (T) | | 0.83 | | | 0.79 | | | |
| Cultivars (C) | | 0.55 | | | 0.52 | | | |
| T × C | | 1.44 | | | 1.37 | | | |

Where T, treatments; C, cultivar; any two means within a column followed by different letters are significantly different, while same letters within a column means non-significant (NS) difference.

followed by cultivars Champion (3%) and Local Khera (4.17%) of the control under light conditions. All the treatments of 2,4-D significantly ($P \leq 0.05$) affected embryogenesis from anthers of medium-sized floral buds (13–15 mm) among various cucumber cultivars (Champion, CP 001, and Local Khera), as shown in Table 4. The treatment of 4 mg $L^{-1}$ 2,4-D was more effective in embryogenesis (10%) in cultivar CP 001 and Local Khera (10%) at the same level of 2,4-D. The minimum embryogenesis (3%) emerged in cultivars CP 001 and Local Khera and Champion (5%) of the control.

***B. 6-Benzylaminopurine (BAP)***

The treatment of 4 mg $L^{-1}$ BAP was more effective in embryogenesis (7.67%) than the control (2.89%) and all other treatments. However, the cultivar Local Khera and CP 001 showed maximum embryogenesis (8% and 9%) at 3 and 4 mg $L^{-1}$, followed by Champion (36%). Meanwhile, the minimum embryogenesis (2%) manifested in the cultivar Champion, followed by Local Khera (2.67%) both under the control treatment. On average, varietal means showed significant ($P \leq 0.05$) differences regarding embryogenesis from anthers of small-sized flower buds (11–13 mm) (Table 4).

Similarly, a significant ($P \leq 0.05$) variation surfaced among various cucumber cultivars (Champion, CP 001, and Local Khera) regarding the effect of varying concentrations of BAP on embryogenesis (%) from anthers of medium-sized flower buds (13–15 mm) (Table 4). The treatment of 4 mg $L^{-1}$ BAP was more effective in embryo formation (5.78%) than the control (1.78%). However, the maximum (7%) embryogenesis was notable in the Local Khera, followed by cultivars Champion (5%) and CP 001 (5.33%).

**Embryogenesis (%) under dark culture conditions**

***A. 2,4-dichlorophenoxyacetic acid (2,4-D)***

The results showed that embryogenesis (%) significantly increased when augmenting the level of 2,4-D (0.5, 1, 1.5, 2, 3, and 4 mg $L^{-1}$) from anthers of tiny flower buds (11–13 mm) under dark culture conditions (Table. 5). The findings detailed that maximum embryo formation (8%) was prominent in cultivar Champion and CP 001, followed by Local Khera (5.67%) at the highest level of 2,4-D (4 mg $L^{-1}$). In contrast, minimum embryogenesis (2%) was evident in Champion (2.17%) and CP 001 (3.33%) in the control. The treatment of 4 mg $L^{-1}$ 2,4-D was more effective for embryogenesis (6.89%) than the control (2.5%) and all other treatments. Similarly, the treatment of 4 mg $L^{-1}$ 2,4-D was superior in embryogenesis (7.73%) in cultivars CP 001 (7%) and Local Khera (6%) at the same level. Conversely, minimum embryogenesis (1.33%) appeared in Local Khera, Champion (2.67%), and CP 001 (3.33%) at the control.

***B. 6-Benzylaminopurine (BAP)***

All the treatments of BAP substantially ($P \leq 0.05$) affected embryogenesis from anthers of small-sized floral buds (11–13 mm) among various cucumber cultivars (Champion, CP 001, and Local Khera) under dark culture conditions (Table 5). The treatment of 4 mg $L^{-1}$ BAP was more effective in embryogenesis (5%) than the control (1.56%) and all other treatments. However, the cultivar Local Khera and CP 001 showed maximum embryogenesis (5.67% and 6%, respectively) at 3 mg $L^{-1}$, followed by cultivar Champion (5.33%) at MS+BAP (4 mg $L^{-1}$). The minimum embryogenesis (1.33%) emerged in cultivar Champion, followed by Local Khera (1.33%) and CP 001 (2%) at the control.

Similarly, a significant ($P \leq 0.05$) variation emerged among cucumber cultivars, Champion, CP 001, and Local Khera, regarding the influence of diverse concentrations of BAP on embryogenesis from anthers of medium-sized floral buds (13–15 mm) under dark culture conditions (Table 5). The treatment of 4 mg $L^{-1}$ BAP was excellent in embryogenesis (4.48%) than the control (1.56%) and all other treatments. However, the cultivar Local Khera produced maximum (5%) embryogenesis, followed by Champion (5%) and CP 001 (4.33%). The interaction between treatments and cultivar proved significant ($P \leq 0.05$) for embryogenesis (%) from anthers of medium-sized floral buds (Table 5).

**DISCUSSION**

*In vitro* calli induction depends on many factors, including the genotype, the donor plant's growing conditions, microspore stage, flower bud pre-treatment, media composition, and culture conditions, such as temperature and available light. Media composition and the concentration of growth regulators in the growing medium are chief factors influencing calli induction and subsequent organogenesis (Maheshwari *et al*., 1982). According to previous reports, the use of various kinds of growth regulators has different regeneration responses under *in vitro* conditions. For instance, the addition of 2,4-D promotes callogenesis, whereas indole-3-acetic acid (IAA) and a-naphthalene acetic acid (NAA) promotes direct embryogenesis (Armstrong *et al*., 1987; Liang *et al*., 1987). This study investigated the callogenesis response of anthers/explants of different cucumber cultivars with varied sizes of floral buds (small and medium) to the application of growth

**Table 5.** Embryogenesis (%) in anther derived calli on MS + 2,4-D and BAP from small and medium size flower buds under dark culture conditions.

| MS medium+ 2,4-D (mg $L^{-1}$) | Embryogenesis (%) under dark culture conditions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Local Khera | Champion | CP 001 | Means | Local Khera | Champion | CP 001 | Means |
| | Small flower size bud (11–13 mm) | | | | Medium flower size bud (13–15) mm | | | |
| Control | 2.00 | 2.17 | 3.33 | 2.50 E | 1.33 k | 2.67 ijk | 3.33 hij | 2.44 E |
| 0.5 | 2.83 | 4.00 | 3.17 | 3.33 DE | 2.17 jk | 3.50 efg | 3.17 efg | 2.95 DE |
| 1.0 | 4.00 | 4.00 | 4.33 | 4.11 CD | 2.67 fgh | 4.16 def | 4.33 cde | 3.72 CD |
| 1.5 | 4.00 | 4.00 | 4.33 | 4.11 CD | 3.83 def | 5.17 bcd | 3.60 efg | 4.20 C |
| 2.0 | 4.33 | 5.00 | 5.00 | 4.78 BC | 6.00 ab | 5.87 abc | 4.50 b-e | 5.46 B |
| 3.0 | 4.67 | 6.00 | 6.00 | 5.56 B | 4.00 def | 6.00 ab | 5.33 bcd | 5.11B |
| 4.0 | 5.67 | 8.00 | 8.00 | 6.89 A | 6.00 ab | 7.00 a | 7.33 a | 6.78 A |
| Means | 3.93 B | 4.74 A | 4.74 A | | 3.64 B | 4.91 A | 4.51 A | |
| LSD *(P ≤ 0.05)* | | | | | | | | |
| Treatments (T) | | 0.91 | | | 0.90 | | | |
| Cultivars (C) | | 0.60 | | | 0.59 | | | |
| T × C | | NS | | | 1.58 | | | |
| MS+ BAP (mg $L^{-1}$) | Local Khera | Champion | CP 001 | Means | Local Khera | Champion | CP 001 | Means |
| | Small flower size bud (11–13 mm) | | | | Medium flower size bud (13–15) mm | | | |
| Control | 1.33 | 1.33 | 2.00 | 1.56 C | 1.00 g | 1.33 fg | 2.33 def | 1.56 C |
| 0.5 | 2.00 | 1.67 | 3.00 | 2.22 C | 2.00 efg | 1.67fg | 4.00 abc | 2.56 B |
| 1.0 | 4.00 | 2.33 | 4.67 | 3.67 B | 3.33 cd | 2.00 efg | 3.33 cd | 2.89 B |
| 1.5 | 5.00 | 2.00 | 5.00 | 4.00 B | 5.00 a | 3.00 cde | 4.00 abc | 4.00 A |
| 2.0 | 5.00 | 3.00 | 5.00 | 4.33 AB | 4.67 ab | 3.67 bc | 3.67 bc | 4.00 A |
| 3.0 | 5.67 | 3.00 | 6.00 | 4.89 A | 4.67 ab | 4.67 ab | 4.00 abc | 4.44 A |
| 4.0 | 5.00 | 4.00 | 6.00 | 5.00 A | 5.00a | 5.00 a | 4.33 cd | 4.48 A |
| Means | 4.00 B | 2.48 C | 4.52 A | | 3.67A | 3.05 B | 3.53 AB | |
| LSD *(P ≤ 0.05)* | | | | | | | | |
| Treatments (T) | 0.76 | | | | 1.25 | | | |
| Cultivars (C) | 0.50 | | | | 0.64 | | | |
| T × C | NS | | | | 2.67 | | | |

Where T, treatments; C, cultivar; any two means within a column followed by different letters are significantly different, while same letters within a column means non-significant (NS) difference.

regulators, such as 2,4-D and BAP in combination with the MS medium under light and dark culture conditions.

Different concentrations of 2,4-D and BAP increased the calli formation percentage in anthers of all selected cucumber cultivars Local Khera, Champion, and CP 001. According to a previous report, applying 2,4-D (2 M) improved the calli formation from anthers of the cucumber cultivar Esfahani (Hamidvand *et al*., 2013). In this study, the bud size also affected the process of calli formation, with increasing bud size reducing the calli formation percentage. On the contrary, Chaar *et al*. (2012) reported that the bud length has no apparent difference in calli formation from the anther culture of the Petunia cv. Purple Wave cultured on a medium supplemented with BA (0.1 mg $L^{-1}$) and NAA (1 mg $L^{-1}$).

According to the report of Ahmad and Spoor (1999), applying NAA and BAP alone and together improved the regeneration ability of Curly Kale (Brassica oleracea). Similarly, the MS media with NAA and BAP at 0.5 mg $L^{-1}$ and 2 mg $L^{-1}$ promoted the calli induction of an ornamental plant, Alstroemeria cv. Fuego (Seyyedyousefi *et al*., 2013). Although the calli formation in Alstroemeria is difficult and time-consuming, supplementing the media with BAP and NAA improved calli formation. Plant growth regulators act as signalling molecules to regulate calli growth (Sundarasekar *et al*., 2012; Seyyedyousefi *et al*., 2013). In this study, the 2,4-D and BAP application also

increased the calli induction rate even at higher doses. These differences might be because varying plant species respond uniquely to growth regulators applied during calli induction. Similarly, the latest study was analogous to the findings of several scientists on calli initiation from another culture of diverse crops, such as watermelon by Silva *et al*. (2021), winter squash and pumpkin (Kurtar *et al*., 2016), summer squash (Mohamed and Refaei, 2004), sweet pepper (Parra-Vega *et al*., 2013), cucumber (Hamidvand *et al*., 2013; Bazargaliyeva *et al*., 2023; Fathurrahman, 2023), and bitter gourd (Usman *et al*., 2015). However, the calli produced under dark culture conditions were exceptionally hard, dry, and brown, while calli developed under light culture conditions were soft and greenish.

Researchers in this study also investigated the effect of modified MS media with 2, 4-D, and BAP on embryogenesis (%) in calli derived from anthers of small (11–13 mm) and medium-sized (13–15 mm) floral buds under light and dark culture conditions. From the results, different cultivars responded in variance when applying growth regulators for embryogenesis. According to a report, somatic embryogenesis emerged when applied with growth regulators in Coffea canephora. It was evident that the media only with cytokinin proved better than with auxins (IBA, IAA, and 2,4-D). In such research, the optimum dose of cytokinin (2-iP, BA, and kinetin) was 5 µM for embryogenesis. Embryo formation occurred only near the cut edges because cytokinin absorption transpired in the cut edges and did not transport to other parts of the explant (Hatanaka *et al*., 1991).

Similarly, in carrot (Daucus carota cv. Kurodagosun), it was noteworthy that removing auxins from the medium improved the embryo formation for the suspension culture of calli. The uniform size of the calli also enhanced embryo formation. In this study, the exogenous application of 2,4-D and IAA inhibited embryo formation, while applying Zeatin promoted embryogenesis. In contrast, benzyl 6-aminopurine (BAP) is also a cytokinin, but it restrained embryogenesis. Zeatin application in combination with 2,4-D and IAA also deterred embryogenesis, observing inhibition. The response of $GA_3$ and ABA did not affect the number of embryos formed compared with the control (Fujimura and Komamine, 1975).

The pertinent study revealed that the percentage of embryogenesis from anther-derived calli under dark conditions was low. However, more embryogenesis occurred in calli developed under light culture conditions. It may be because the calli produced under dark culture conditions were durable and dry, while calli developed under light culture conditions were soft and greenish. Similar findings also came from Medina *et al*. (1998). Previous studies have reported the occurrence of abnormal but embryo-like structures growing on the calli surface in squash (Kurtar *et al*., 2016), cucurbit interspecific hybrids (Rakha *et al*., 2012), bitter gourd (Usman *et al*., 2015), hardwood species (Corredoira *et al*., 2019), and in Cork oak (Ali *et al*., 2023). Earlier work has reported that variations of cucumber tissues required an appropriate ratio of auxin and cytokinin (Lotfi *et al*., 2003; Plapung *et al*., 2014). Our results align with the findings of Kubaláková *et al*. (1996), who reported the aging of cucumber calli cultures, an increase in polyploidy, and a decrease in the regeneration ability in cucumber calli culture. Similarly, no emergence was evident in the anther culture (Rakha *et al*., 2012).

Based on the study results, adding growth regulators 2,4-D and BAP can be favorable to improve the anther-derived calli formation percentage, reduce the time required for calli formation, and limit success in embryogenesis. However, further systematic studies are necessary to understand the underlying mechanisms. Gene expression analysis and genome-wide transcription profiling can further help to underpin the key regulators involved in the process of embryogenesis from anther derived calli.

## CONCLUSIONS

Application of the highest level of 2,4-D and BAP at 4 mg $L^{-1}$ was most effective for calli induction and embryogenesis. However, more calli induction was prominent under dark

culture conditions, while maximum embryo formation occurred under light culture conditions.

## ACKNOWLEDGMENTS

The authors are thankful to Plant Tissue Culture Cell, University of Agriculture, Faisalabad for providing the facilities for current research. The authors are also thankful to Vegetable Research Institute, Ayub Agricultural Research Institute, Faisalabad for providing the seeds of cucumber cultivars for current research work.

## REFERENCES

Abdollahi MR, Najafi S, Sarikhani H, Moosavi SS (2016). Induction and development of anther-derived gametic embryos in cucumber (*Cucumis sativus* L.) by optimizing the macronutrient and agar concentrations in culture medium. *Turk J. Biol*. 40: 571–579. https://api.semanticscholar.org/CorpusID:89524921.

Abo-Shama HM, Atwa MM (2019). Anther culture in potato (*Solanum tuberosum* L.) in vitro. *J. Plant Biochem. Physiol*. 7:244. doi: 10.35248/2329-9029.19.7.244.

Ahmad S, Spoor W (1999). Effects of NAA and BAP on callus culture and plant regeneration in Curly Kale (*Brassica oleracea* L.). *Pak. J. Bio. Sci*. 2 (1): 109–112. https://doi.org/10.3923/pjbs.1999.109.112.

Ali NB, Benkaddour R, Rahmouni S, Boussaoudi I, Hamdoun O, Hassoun M, Azaroual L, Badoc A, Martin P, Lamarti A (2023). Secondary somatic embryogenesis in Cork oak: Influence of plant growth regulators. *Forest Sci. Technol.* 19(1):78–88. https://doi.org/10.1080/21580103.2023.2172462.

Armstrong KC, Metz SG, Mascia PN (1987). Two-regeneration system for the production of haploid plants from wheat anther culture. *Plant Sci*. 51: 231–237. https://doi.org/10.1016/0168-9452(87)90197-X.

Bazargaliyeva A, Utarbayeva N, Nussupova A, Admanova G, Yechshanova G, Kuanbay Z, Sarzhigitova A, Baubekova A (2023). Ecological varietal evaluation of cucumber (*Cucumis sativus* L.) under field conditions. *SABRAO J. Breed. Genet*. 55(1): 90-96. http://doi.org/10.54910/sabrao2023.55.1.8.

Bokhari SA, Fatima B, Usman M, Khan MS, Rashid B (2023). Embryogenic responses in anther derived calli of white and pink flesh guava (*Psidium guajava* L.) cultivars. *Pak. J. Agri. Sci.* 60(4): 537–46. https://doi.org/10.21162/PAKJAS/23.72.

Chaar M, Pinker I, Grieger P, Bohme M (2012). The effect of flower bud size and pre-treatment on calli induction and plant regeneration in anther culture of petunia 'Purple Wave'. *Acta Hortic*. (961): 375–382. https://doi.org/10.17660/ActaHortic.2012.961.49.

Corredoira E, Merkle SA, Martınez MT, Toribio M, Canhoto JM, Correia SI, Ballester A, Vieitez AM (2019). Non-zygotic embryogenesis in hardwood species. *Crit. Rev. Plant Sci*. 38(1): 29–97. https://doi.org/10.1080/07352689.2018.1551122.

Domblides EA, Belov SN, Soldatenko AV, Pivovarov VF (2019). Production of doubled haploids in cucumber. *Vege. of Russia.* 5: 3–14. https://doi.org/10.18619/2072-9146-2019-5-3-14. (in Russian).

Fathurrahman F (2023). Growth and genetic characteristics of cucumber (*Cucumis sativus* L.) Cultivar mercy f1 hybrid and mutant populations. *SABRAO J. Breed. Genet*. 55(2): 485-494. http://doi.org/10.54910/sabrao2023.55.2.20.

Fujimura T, Komamine A (1975). Effects of various growth regulators on the embryogenesis in a carrot cell suspension culture. *Plant Sci. Lett*. 5(6): 359–364. https://doi.org/10.1016/0304-4211(75)90001-2.

Gałązka J, Niemirowicz-Szczytt K (2013). Review of research on haploid production in cucumber and other cucurbits. *Folia Hort*. 25(1): 67–78. https://doi.org/10.2478/fhort-2013-0008.

GOP (Government of Pakistan) (2024). Agricultural Statistics Yearbook. Ministry of National Food Security and Research, Islamabad, Pakistan.

Hamidvand Y, Abdollahi MR, Chaichi M, Saeed S (2013). The effect of plant growth regulators on callogenesis and gametic embryogenesis from anther culture of cucumber (*Cucumis sativus* L.). *Agric. Food Sci*. 5 (10): 1089–1095. https://doi.org/10.22059/ijhs.2017.230756.1223.

Hatanaka T, Arakawa O, Yasuda T, Uchida N, Yamaguchi T (1991). Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. *Plant Cell Rep*. 10(4): 179–82. https://doi.org/10.1007/BF00234290.

Jaskani MJ, Kwon SW, Koh GC, Huh YC, Ko BR (2004). Induction and characterization of tetraploid watermelon. *J. Kor. Soc. Hort.*

*Sci.* 45(2): 60–65. https://doi.org/10.3390/plants8100419.

Kaur A, Reddy MS, Kumar A (2018). Direct somatic embryogenesis of potato (*Solanum tuberosum* L.) cultivar 'Kufri Chipsona 2'. *Plant Cell Tiss. Org. Cult.* 134(3):457–466. https://doi.org/10.1007/s11240-018-1435-4.

Kubaláková M, DoleŽel J, Lebeda A (1996). Ploidy instability of embryogenic cucumber (*Cucumis sativus* L.) callus culture. *Biol. Plant.* 38, 475–480. https://doi.org/10.1007/BF02896685.

Kurtar ES, Balkaya A, Kandemir D (2016). Evaluation of haploidization efficiency in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) through anther culture. *Plant Cell Tiss. Organ. Cult.* 127(2):497–511. https://doi.org/10.1007/s11240-016-1074-6.

Lazarte JE, Sasser CC (1982). Asexual embryogenesis plantlet development in anther culture of *Cucumis sativus* L. *HortScience* 17:88.

Liang GH, Xu A, Tang H (1987). Direct generation of wheat haploids via anther culture. *Crop Sci*. 27: 336–339. https://doi.org/10.2135/cropsci1987.0011183X002700020043x.

Lotfi M, Alan AR, Henning MJ, Jahn MM, Earle ED (2003). Production of haploid and doubled haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance. *Plant Cell Rep*. 21: 1121–1128. https://doi.org/10.1007/s00299-003-0636-3.

Maheshwari SC, Rashid A, Tyagy AK (1982). Haploid from pollen grains-retrospect and prospect. *Amer J. Bot*. 69:865–879. http://www.jstor.org/pss/2442979.

Malepszy S (1988). Cucumber (*Cucumis sativus* L.). In: Y.P.S. Bajaj (ed.), Crops II. Biotechnology in Agriculture and Forestry, Vol 6. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-73520-2_13.

Medina M, Villalobos NP, Cruz A, Guerra H (1998). Effect of culture medium and light conditions on the morphological characteristics and carbohydrate contents of *Medicago strasseri* calli. *Acta Physiol Plant*. 20(4) 383–392. https://doi.org/10.1007/s11738-998-0024-2.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*. 15,473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Parra-Vega V, Renau-Morata B, Sifres A, Seguı´-Simarro JM (2013). Stress treatments and *in vitro* culture conditions influence microspore embryogenesis and growth of calli from anther walls of sweet pepper (*Capsicum annuum* L.). *Plant Cell Tiss. Organ Cult*. 112(3): 353–360. https://doi.org/10.1007/s11240-012-0242-6.

Plapung P, Khumsukdee S, Smitamana P (2014). Development of cucumber lines resistant to Cucumber mosaic virus by ovule culture. *Int. J. Agri. Technol.* 10: 733–741.

Rakha MT, Metwally EI, Moustafa AS, Etman A, Dewir YH (2012). Production of cucurbita interspecific hybrids through cross pollination and embryo rescue technique. *World Appl Sci J*. 20(10): 1366–1370. 10.5829/idosi.wasj.2012.20.10.299.

Seyyedyousefi SR, Behzad K, Naghi PD, Ali S (2013). Calli induction in alstroemeria using NAA and BAP. *Eur. J. Exp. Biol.* 3(5):137–140.

Silva CM, Dias RCS, Melo NF (2021). The effect of temperature and growth regulators on calli induction in watermelon anthers. *Braz Arch Biol Technol*. 64: 1–9. https://doi.org/10.1590/1678-4324-2021180505.

Sreenayana B, Nakkeeran S (2019). Effect of oil based formulation of *Trichoderma* spp. on growth parameters of cucumber seedlings. *Int. J. Curr. Microbiol. Appl. Sci*. 8(8):200–209.

Steel RGD, Torrie JH, Dicky DA (1997). Principles and Procedures of Statistics. A Biological Approach. McGraw Hill Book Co. New York. pp. 336–354.

Sundarasekar J, Anthony JJJ, Murugaiyah V, Subramaniam S (2012). Preliminary responses of 2,4-D and BAP on calli initiation of an important medicinal-ornamental *Hymenocallis littoralis* plants. *J. Med. Plant Res*. 6(11): 2088–2093.10.5897/JMPR11.790.7

Usman M, Hussain Z, Fatima B (2011). Somatic embryogenesis and shoot regeneration induced in cucumber leaves. *Pak. J. Bot*. 43(2): 1283–1293.

Usman M, Bakhsh K, Fatima B, Zaman Q, Shah MH(2015). Exploring embryogenic competence in anthers of bitter gourd (*Momordica charantia* L.) cultivar Faisalabad Long. *J. Anim Plant Sci*. 25(1): 181–188.

Wernsman EA (1992). Varied roles for the haploid sporophyte in plant improvement. In: H.T. Stalker, and J.P. Murphy (eds.), Plant Breeding in the 1990s. *CABI International, Wallingford, UK*, pp. 461–484.