Stimulation of Androgenesis in Cabbage (*Brassica olecracea var* Capitata) Anthers cultivated in Vitro by Growth Regulators and Medium Sucrose Concentration.

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Abstract: Induced androgenesis of cabbage anthers using BAP in combination with NAA and 2,4- D, and different sucrose concentrations were investigated. The experiment was conducted in 2013 at the lab of plant tissue culture, Department of Arid Land Agriculture, King Abdullaziz University, Saudi Arabia. Anthers at the stage of microsporogenesis of the first formed flower buds and the MS Basel media supplemented with AC ($0.5gL^{-1}$) were used. The culture medium was supplemented with BAP in combination with NAA and 2,4- D and different sucrose concentrations. The culture medium MS-7 ($1mgl^{-1}$ BAP + $0.5mgl^{-1}$ 2, 4- D) enhanced 90% of the cultured anthers to form embryos followed by MS-8 ($1mg.l^{-1}$ BAP + $1mgl^{-1}$ 2,4- D) with 89.67%. The MS supplemented with $5mgl^{-1}$ BAP + $1mgl^{-1}$ NAA (MS-2) prevented the development of cabbage anthers. The formed embryos were stimulated to develop callus and plantlets on the medium supplemented with lower concentration of BAP and 2,4- D (MS-7 and MS-8). The MS-7 medium improved 49.60% of the formed embryos to develop callus and 27.60% to plantlets. Percentage of embryos that formed callus on MS-8 medium was 51.82% of which 19.87% developed plantlets. Higher concentration of BAP in the culture medium decreased percentage of embryos producing plantlets. Higher percentage (89 and 90%) of anthers induced embryos and embryos formed plantlets (34.31 and 26.67%) were stimulated on MS medium with lower sucrose concentration ($20gl^{-1}$ and 30 gl^{-1}). Increasing sucrose concentration to $50gl^{-1}$ in the culture media enhanced embryos to develop callus and reduced the percentage of embryos developing into plantlets.

[Magdi Ali Ahmed Mousa, Ahmed Abduallah Said Bakhashwain and Mohamed Abdul Raheem Shaheen. **Stimulation of Androgenesis in Cabbage (Brassica olecracea var Capitata) Anthers cultivated in Vitro by Growth Regulators and Medium Sucrose Concentration.** *J Am Sci* 2014;10(2):174-180]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 26. doi:10.7537/marsjas100214.26

Key words: BAP, NAA, 2,4- D, sucrose, androgenesis, cabbage, anther culture.

Abbreviations: BAP/BA: 6-Benzylaminopurine, **NAA**: Naphthalene Acetic Acid, **2,4- D:** 2,4-Dichlorophenoxyacetic, **MS**: Murashige and Skoog, **µM**: Micro-Mole, **PGRs**: Plant growth regulators, **B5**: Gamborg B5 Medium, **CRD:** Completely Randomized Design
AC: Activated Charcoal, mgl^{-1} and gl^{-1}: milligram and gram per litter.

1. Introduction

Recently, seed companies paid greater attention to cabbage (*Brassica olecracea* var *capitata*) crop 2n=2x= 20 due to its effective therapeutic potential to reduce incidence of certain cancers including lung, colon, breast, ovarian and bladder cancers (Higdon *et al.,* 2007; Zhao *et al.,*2007; Yuan *et al.,* 2011). Breeding new hybrids of cabbage with high yield and quality required a minimum of 7 years and intensive labor due to the bigger genome (2n=2x= 20) and cross pollination behavior of the plants (Rudolf *et al.,* 1999; Gorecka and Krzyzanowska *et al.,* 2007). High genetically uniform lines/cultivars (i.e. pure line and homozygote line) with desired traits are urgently required before starting breeding program. Anther culture is an effective tissue culture technique that was frequently used for the production of homozygote lines of numerous crops. There are three steps for the production of double haploid cabbage plants using anther culture: a) initiating androgenetic embryos in anthers, b) regenerating haploid plantlet from androgenetic embryos, and c) doubling the chromosome number of the haploid plants (Zhao *et al.,* 1995; Palmer *et al.,* 1996; Gorecka and Krzyzanowska, 2007). Obtaining androgenic plants from Brassica crops was reported in broccoli (Duijs *et al.,* 1992; Lee and Nam 1995; Yuan *et al.,* 2011), Brussels sprouts (Biddington *et al.,* 1993; Ockendon and McClenaghan, 1993) and cauliflower (Chauvin *et al.,* 1993; Stipic and Campion, 1997). In cabbage, anther and microspore cultures are rarely used in breeding programs probably due to the difficulty to obtain androgenic lines with good quality, uniformity and stability in consecutive generations (Higdon *et al.,* 2007; Zhao *et al.,* 2007; Yuan *et al.,*2011). Production of androgenric cabbage plants through anther cultures

may be improved by adding ethylene antagonist silver nitrate (AgNo3) to the culture medium (Biddington *et al.,* 1988), combined cold and heat shock pretreatments of the flower (Yuan *et al.,* 2011), and addition of activated charcoal to the culture medium (Duijs *et al.,* 1992). The present study aimed to stimulating the androgenesis of cabbage anthers and regeneration of androgenic embryos by BAP in combination with NAA and 2, 4- D and different sucrose concentrations in the culture medium.

2. Materials and Methods

1. Plant Materials and flower bud sterilization

This experiment was conducted in 2013 at the lab of plant tissue culture, department of Arid Land Agriculture, Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdullaziz University, Saudi Arabia. The first formed flower buds of 2.5-5 mm were collected from the donor plants for anther culture. The donor plants belong to the local cabbage cv. 'Balady', which is commonly grown in autumn and winter seasons in different regions of Saudi Arabia. The stage of microsporogenesis was determined by microscopic observation of crushed specimens in hematoxyline (Fig 2A). The flower buds were left under running tap water for 30 min, then buds were transferred to the laminar flow hood and rinsed in 70% ethanol for 1 min followed by rinsing in 10% Clorox for 10 min and finally buds were washed four times in deionized water.

2. Culture Media and growing conditions

The Murashige and Skoog Basel medium (MS) supplemented with 0.5gL^{-1} activated charcoal (AC) was used. The medium pH was adjusted to 5.8 ± 0.1 prior to add agar and the medium were autoclaved at 121^{o}C and 1.05 kg cm^{-2} for 15 min. Thermo-labile vitamins and growth regulators were add to the autoclaved medium through membrane filters (Millex-GS 0.20 µm filter unit) and the . Medium were alliquoted (10ml) into sterilized petri dishes. Ten anthers were cultured in each petri dish and the petri dishes subsequently were plugged and bundled with a layer of parafilm (Pechiney Plastic Packaging, Chicago, IL. 60631). The petri dishes with anthers were incubated under 16-h lights (white fluorescent light with intensity of 55 µmol $m^{-2}s^{-1}$) and 8-h dark at 24 ± 1^{o}C.

3. Stimulation and regeneration of androgenic embryos

3.1 Experiment of plant growth regulators (PGRs)

Three BAP and two NAA and 2,4- D concentrations were used in combination for the stimulation and regeneration of androgenic embryos of cabbage anthers. The experiments were laid out in Completely Randomized Design (CRD) using 3 replicates with 5 petri dishes of each treatment (15petri dishes per treatment over the 3 replicate). The applied BAP, NAA and 2,4- D treatments were as following: 1) MS-1 (1mgl^{-1} BAP + 0.5mgl^{-1} NAA), 2) MS-2 (1mg.l^{-1} BAP + 1mg.l^{-1} NAA), 3) MS-3 (3mg.l^{-1} BAP + 0.5mg.l^{-1} NAA), 4) MS-4 (3mg.l^{-1} BAP + 1mg.l^{-1} NAA), 5) MS-5 (5mgl^{-1} BAP + 0.5mgl^{-1} NAA), 6) MS-6 (5mgl^{-1} BAP + 1mgl^{-1} NAA), 7) MS-7 (1mgl^{-1} BAP + 0.5mgl^{-1} 2,4- D), 8) MS-8 (1mgl^{-1} BAP + 1mgl^{-1} 2,4- D), 9) MS-9 (3mgl^{-1} BAP + 0.5mgl^{-1} 2,4-D), 10) MS-10 (3mgl^{-1} BAP + 1mgl^{-1} 2,4- D), 11) MS-11 (5mgl^{-1} BAP + 0.5mgl^{-1} 2,4- D) and 12) MS-12 (5mgl^{-1} BAP + 1mgl^{-1} 2,4- D).

3.2 Experiment of sucrose concentration

This experiment was carried out to study the effects of medium sucrose concentration on stimulation and regeneration of cabbage anthers. Four sucrose concentrations 20gl^{-1}, 30gl^{-1}, 40gl^{-1} and 50gl^{-1} were added to the culture medium MS-7 (1mgl^{-1} BAP + 0.5mgl^{-1} 2,4- D) and MS-8 (1mgl^{-1} BAP + 1mgl^{-1} 2,4- D). A Completely Randomized Design (CRD) using 3 replicates (15 dishes per treatment with total of 120 Petri dishes over the experiment was used. The following sucrose concentrations were tested: 1) MS-13 (20gl^{-1} Sucrose +1mgl^{-1} BAP + 0.5mgl^{-1} 2,4- D), 2) MS-14 (20gl-1+1mgl^{-1} BAP + 1mgl^{-1} 2,4- D), 3) MS-15 (30g l^{-1} sucrose + 1mgl^{-1} BAP + 0.5mgl^{-1} 2,4- D), 4) MS-16 (30gl^{-1} sucrose + 1mgl^{-1} BAP + 1mgl^{-1} 2,4-D), 5) MS-17 (40gl^{-1} sucrose + 1mgl^{-1} BAP + 0.5mgl^{-1} 2,4- D), 6) MS-18 (40gl^{-1} sucrose + 1mgl^{-1} BAP + 1mgl^{-1} 2,4- D), 7) MS-19 (50gl^{-1} sucrose + 1mgl^{-1} BAP + 0.5mgl^{-1} 2,4- D) and 8) MS-20 (50gl^{-1} sucrose + 1mgl^{-1} BAP + 1mgl^{-1} 2,4- D).

4. Data collected:

The following parameters were assessed in both experiments from clean cultures: a) % of anthers developed embryos, b) % embryos formed callus and c) % embryos formed plantlets.

Data analysis

Analysis of variance related to CRD experiments as described by Gomez and Gomez, (1984), was conducted. The treatment means were compared by the Least Significant Differences test (LSD) at 5% probability level.

3.Results

Effects of plant growth regulators (PGRs)

There were observed significant differences between the applied growth regulator combinations on the stimulation of androgenesis and regeneration of androgenic embryos of cabbage anthers (Table 1). Androgenesis and regeneration of androgenic embryos were significantly enhanced by the application of BAP and 2,4- D comparing BAP with NAA. The MS-7 culture medium with low BAP + 2,4- D (1mgl^{-1} BAP + 0.5mgl^{-1} 2,4- D) and MS-8 (1mgl^{-1} BAP + 1mgl^{-1} 2,4- D) stimulated higher percentage of anthers to

form embryos (90% of total cultured anthers) followed by MS-8 (1mg.l^{-1} BAP + 1mg.l^{-1} 2,4- D) with 89.67% (Figures 1A and 2B&C). Increasing BAP in the culture medium restricted the percentage of anthers performed embryos in spite of 2,4- D concentration. Contrary, the androgenesis of cabbage anthers were restricted on the culture medium supplemented with low BAP and NAA. The medium MS-2 (1mgl^{-1} BAP + 1mgl^{-1} NAA) enhanced 39% of total cultured anthers to form embryos followed by MS-1 (1mgl^{-1} BAP + 0.5% mgl^{-1} NAA) with 43.3% (Fig 1A). Significant increase in percentage of anthers induced embryos was observed with the increase of BAP in the culture medium despite of NAA (MS-3, MS-5 and MS-4 with 68.33%, 63.33% and 52.67% of total cultured anthers, respectively). However, the cabbage anthers were not stimulated on culture medium MS-6 (5mgl^{-1} BAP + 1mgl^{-1} NAA) (Figures 1A&2D). The regeneration of androgenic embryos was significantly affected by the applied growth regulator. Also, the culture medium supplemented with combinations of BAP+2,4- D significantly increased percentages of regenerated embryos comparing BAP+NAA. The culture medium with low BAP+2,4- D produced the highest percentages of embryos regenerated callus and plantlets. The percentages of regenerated callus and plantlets were 49.60% and 26.7% of total cultured embryos on MS-7 and 51.82% and 19.67% on MS-8 (Figures 1B&C and 2E-I). Replacement of 2,4- D by NAA in the culture medium significantly reduced the regeneration of androgenic embryos with all tested culture medium except MS-4 (3mgl^{-1} BAP + 1mgl^{-1} NAA) and MS-5 (5mgl^{-1} BAP + 0.5mgl^{-1} NAA (Figures 1B&C). The least percentages of embryos formed callus and plantlets were 13.80% and 2.02% and were produced by MS-5 and MS-4, respectively. Increasing BAP from 1 to 5 mg.l^{-1} in the culture medium significantly reduced the regeneration of androgenic embryos to callus and plantlets with both 2,4- D and NAA.

Effects of medium Sucrose concentrations

The stimulation and regeneration of cabbage anthers were significantly affected by the applied medium sucrose concentrations (Table 2) and (Figures 3A-E).The results showed that low medium sucrose concentration obviously stimulated the androgenesis of cabbage anthers compared to high sucrose concentration (Fig 3A). The culture medium MS-15 and MS-16 (with 30g.l^{-1} sucrose) stimulated 90% and 89.67% of the total cultured anthers to form embryos followed by MS-14 and MS-13 (20gl^{-1}) with 89.00% and 82.00%, respectively (Fig 3A). Elevated sucrose concentration in the culture medium caused significant reduction in the percentage of induced anthers (Table 2). The least percentages of anthers that formed embryos were 23.33% and 35% and were produced by MS-20 and MS-19 (supplied with 50gl^{-1} Sucrose) (Fig 3A). As presented in Fig 3B, transfer of the formed embryos to culture medium supplemented with 50g.l^{-1} sucrose (MS-20 and MS-19) enhanced 83.01% and 82.82%of the embryos to regenerate callus. Moreover, high sucrose concentration in the culture medium restricted the regeneration of embryos to plantlets. The least percentages of embryos formed plantlets were 2.64% and 7.3% and were produced on MS-20 and MS-19 (Fig 3C). On the contrary, the embryos were cultured on medium supplemented with low sucrose concentration (20gl^{-1} and 30gl^{-1}) regenerated lower percentages of callus (40.14%, 46.30%, 49.63% and 51.82% of total cultured embryos on MS-17, MS-13, MS-14 and MS-18, respectively) (Fig 3C). Additionally, Low medium sucrose concentration stimulated higher percentages of embryos to regenerate plantlets (34.31% for MS-13 and 30.27% for MS-17) compared to 2.64% for MS-20 and 7.30% for MS-19 (medium supplemented with 50gl^{-1} sucrose) (Fig 3C).

Table (1): Mean squares for the effects of growth regulator (GR) combination on the stimulation and regeneration of cabbage anther.

Effects of Growth Regulators				
Source	df	% anthers developed embryos	% embryos formed callus	% embryos formed plantlet
Rep	2	142.53	169.67[2]	20.83[2]
GR[1]	11	1998.45***	557.25**	183.14***
Error	22	21.56	168.26	23.47

[1] GR = Growth regulators

[2] The degrees of freedom for growth regulator (GR) and Error are 10 and 20 because the exclusion of the culture medium MS-6 from further analysis.

Table (2): Mean squares for the effects of medium sucrose concentration on the stimulation and regeneration of cabbage anther.

Effects of Medium Sucrose Concentration				
Source	df	% anthers developed embryos	% embryos formed callus	% embryos formed plantlet
Rep	2	13.54	2.14	10.77
Sucrose	7	2089.90***	901.18***	373.35***
Error	14	36.64	62.46	27.45

[1] GR = Growth regulators

[2] The degrees of freedom for growth regulator (GR) and Error are 10 and 20 because the exclusion of the culture medium MS-6 from further analysis.

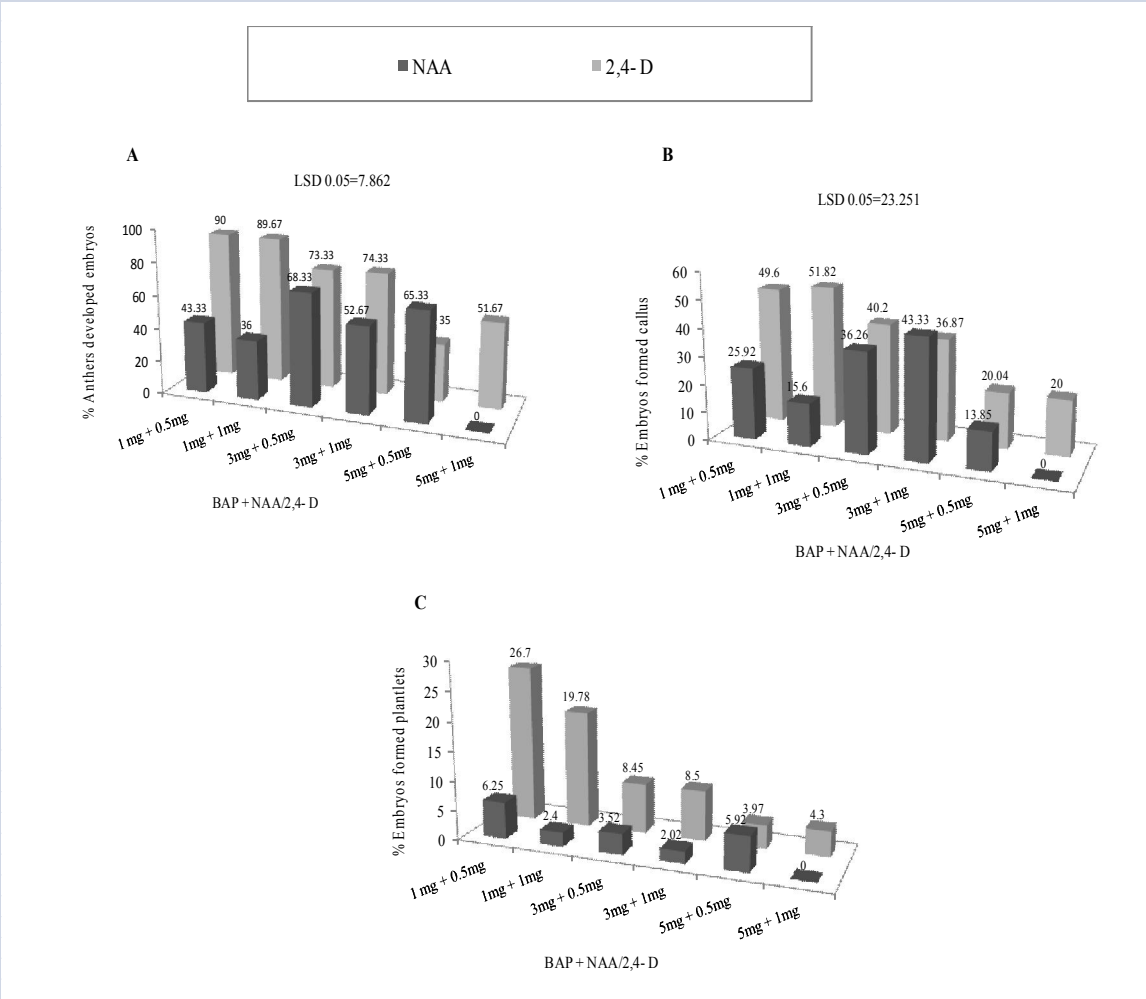

Fig (1): Androgenesis of cabbage anther on Murashige and Skoog basal medium (MS) supplemented with 0.5g activated charcoal (AC) and different growth regulator (GR) combination: A) % anthers developed embryos, B) % embryos formed callus and C) % embryos formed plantlets.

4.Discussion

The effects of growth regulator (GR) and medium sucrose concentration on the stimulation androgenesis of cabbage (*Brassica oleracia* var *capitat*) anther were investigated. Culture medium with low BAP and 2,4- D concentrations (MS-7 and MS-8) resulted in the maximum percentages of anthers that developed to embryos, embryos that developed callus and embryos that developed plantlets. These results were in line with that observed by Górecka *et al.* (2007). They found that MS medium with 1 mgl^{-1} BA obtained the maximum percentages of embryos formed shoots (8.3%), embryos formed callus (25%), and embryos developed into plants (25%), while B5-1 a medium without amino acids and hormones, containing 20 g/L sucrose resulted in the lowest percentages of embryos that formed shoots, embryos that formed callus, and embryos that developed into plants. Dore and Boulidard (1988) found that embryos on a medium without growth substances transformed into single complete plants, whereas from embryos placed on a medium with 0.1 mgl^{-1} BA shoots emerged. Krzyżanowska *et al.* (2006) reported that MS with 20 gl^{-1} sucrose, 1mg.L-1 BA and 0. gl^{-1} NAA showed the highest percentages of embryos producing callus (47.9%) and embryos producing shoots (15.1%) comparing to B5 without amino acids and hormones and B5 without amino acids 20 mg.L-1 kinetin. George *et al.* (2008) and Ravanfar *et al.* (2009) observed that BAP was most effective in enhancing shoot multiplication and elongation. Also, they reported that BAP promoted differentiation of cell into shoot initials followed by the formation of shoots. The high concentrations of BAP in combinations with 0.5mgl^{-1} and 1mgl^{-1} 2,4- D and NAA produced the maximum percentages of undeveloped anthers and dead embryos. This may be attributed to the negative and toxic effects of the higher concentrations of BAP

on anthers induction and embryos development. Ravanfar *et al.* (2009) reported that above 5 mgl^{-1} BAP the mean number of shoots formed per explant decreased and became toxic to shoot growth. Regarding to sucrose concentration, the MS media supplemented with $20gl^{-1}$ and $30gl^{-1}$ sucrose concentration should be the superlative results of all assessed parameters with no significant differences except for percentage of embryos formed plantlets. The MS medium supplemented with high sucrose concentration produced the maximum percentages of undeveloped anthers and embryos forming callus, and the minimum percentage of embryos forming plantlets. Krzyżanowska *et al.* (2006) found that MS with 20 gl^{-1} sucrose showed the highest percentages of embryos producing callus (47.9%) and embryos producing shoots (15.1%). Roulund *et al.* (1990) studied the effects of sugar concentrations and types on anther culture of head cabbage. They found higher average response on the sucrose media (3 .4 embryos/100 anthers) compared to the media with maltose (1.6 embryos/100 anthers). The highest concentration of sugars (13%) was generally superior to 10 and 7% for embryo formation. Zhang *et al.* (2006) observed that the optimal medium for embryo induction of cabbage anthers was B_5 + 2.0 mgl^{-1} 2,4-D+2.0 mg^{-1} KT+6% sucrose.

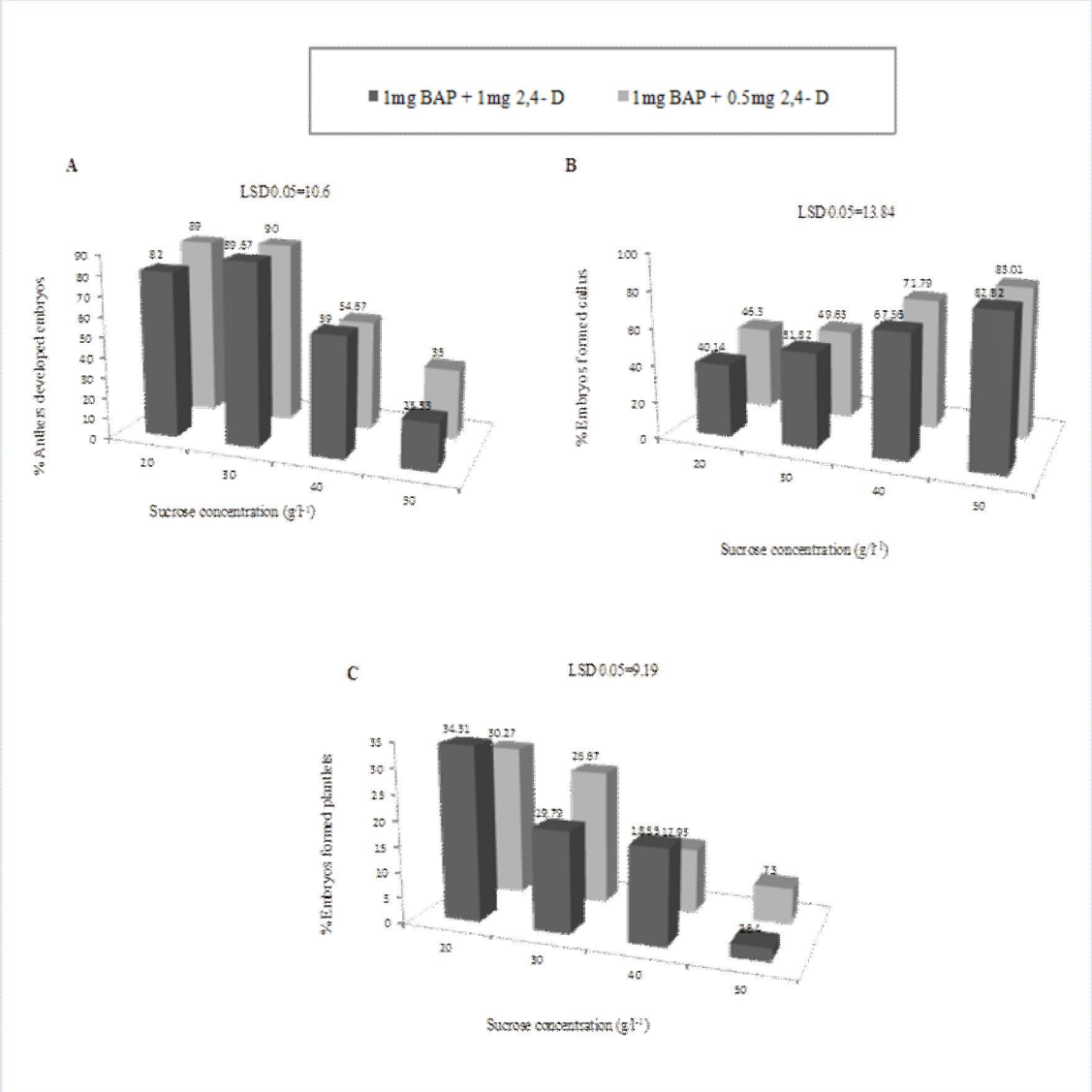

Fig (3): Effects of medium sucrose concentration on the androgenesis of cabbage anther. The Murashige and Skoog basal medium (MS) supplemented with 0.5g activated charcoal (AC) and four sucrose concentration were used: A) % anthers developed embryos, B) % embryos formed callus and C) % embryos formed plantlets.

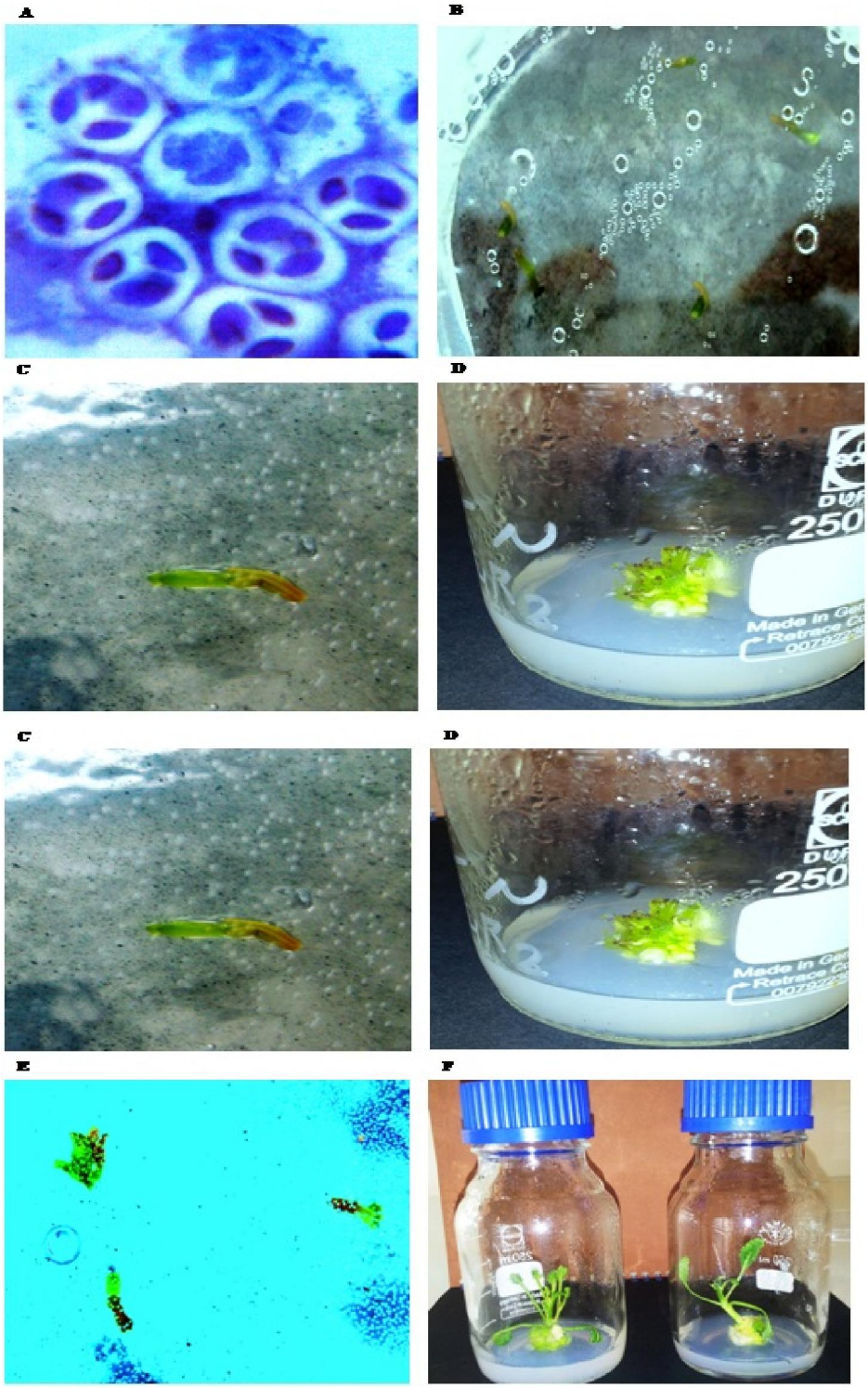

Fig (2): Effects of growth regulator (GR) and medium sucrose concentration on the androgenesis of cabbage anther. The Murashige and Skoog basal medium (MS) supplemented with 0.5g activated charcoal (AC) was used: A) The stage of microsporogenesis, B and C) anthers developed embryos, D) embryos formed callus, E) embryos formed plantlets and F) cabbage plants derived from anther culture.

Conclusion

The studied growth regulator (GR) combinations and medium sucrose concentrations differed markedly in their effects on androgenesis of cabbage anthers. BAP in combination with 2,4- D increased the callus induction and embryos development either to callus or plantlets. The lower concentrations of sucrose were superior in inducing anthers and embryos developed to callus and plantlets.

Acknowledgement

This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant No (231/155/1433). The authors, therefore, acknowledge with thanks the DSR technical and financial support.

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2/19/2014