Research Article

Ekin
Journal of Crop Breeding and Genetics

3(2):82-87 2017

www.ekinjournal.com

Ekin International biannual peer-reviewed journal

Anther Culture in Red Cabbage (*Brassica oleraceae* L. var. *capitata* subvar. *rubra*): Embryogenesis and Plantlet Initiation

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Citation:
Cilingir A., Dogru S. M., Kurtar E. S., Balkaya A., 2017. Anther Culture in Red Cabbage (*Brassica oleraceae* L. var. *capitata* subvar. *rubra*): Embryogenesis and Plantlet Initiation. Ekin J. 3(2):82-87.

Received: 11.05.2017 ***Accepted: 19.06.2017*** ***Published Online: 29.07.2017*** ***Printed: 31.07.2017***

ABSTRACT

Cabbages, cauliflower and broccoli breeding activities are very low level compared with the other vegetable species. This study was conducted to determine the efficacy of the anther culture technique on the in vitro embryogenesis and according plantlet initiation and to the author's knowledge, this is the first reports in red cabbage species. The anthers at uninucleate microspore stage (about 3-5 mm long) were collected at florescence times and then cultured on a solid MS and B5 medium with the addition of 2,4-D, NAA, Glutamine, Serine, Silver nitrate, IAA, BAP and different sucrose concentration to induce callogenesis, embryogenesis, and plantlet initiation. The culture medium, sucrose concentration, and genotype were found to be highly effective on androgenesis. Among the genotype, Zencibas cv. produced the best result with a combination of M medium+30 g/l sucrose for embryogenesis and plantlet initiation, while, Integro F_1 and Cabellero F_1 did not generate an androgenic response. This results can assist cabbage breeders to develop new hybrid cultivars in breeding programs.

Keywords: Red cabbage, anther culture, embryogenesis, plantlet initiation.

Introduction

Brassica vegetables are an important and highly diverse group of crops grown world-wide that belong mainly to the species *Brassica oleracea* and *Brassica campestris* (Monteiro and Lunn, 1998; Balkaya *et al*., 2005). Red cabbage is a member of the genus *Brassica* within the economically important *Brassicaceae* family. Cabbages breeding studies have been increased the last years according to the other *Brassica* vegetable species (Balkaya *et al*. 2016). Thus, it is well known that all *Brassica* spp. are open pollinated and improve new F_1 cultivars with desirable agronomic traits need to many times and also intensive efforts. Moreover, red cabbage is a biennial crop for seed production, and selfing depression, incompatibility and male sterility are seen in some genotypes, frequently (Dogru and Balkaya, 2015). Thus, doubled haploid (DH) technology is a unique solution and a new tool to the recovery of genetically uniform parental lines for the release of hybrid varieties in a short time (Kurtar and Balkaya, 2010; Dogru *et al*., 2016). These valuable DH lines could be obtained from anther-microspore culture, ovule-ovary culture and pollen irradiation in many species.

Androgenesis is an effective and widely used technique for the generating homozygote haploid lines of numerous species. Various techniques for microspore/anther culture have been developed for *Brassica* species (Cardoza and Stewart, 2004). The first successful production of haploid plants via androgenesis was reported in broccoli by Keller and Armstrong (1983), in Brussels sprouts by Ockendon (1984) and in white head cabbage by Kameya and Hinata (1970).

To our recent knowledge, there are no reports about androgenesis in red cabbage species via anther culture. Thus, this study was conducted to determine the frequency of anther culture for our future breeding efforts, firstly. So, the effects of medium, genotype and sucrose concentration were primarily investigated.

Materials and methods

Materials, preparation, and culture

In this study, the three red cabbage cultivars named open pollinated local variety, commercial Zencibas, Integro F_1 and Cabellero F_1 were used for anther culture process. The seeds of donor lines were sown in plastic flats (cell volume 80 cm^3 and 45 cells per flat) containing peat-moss. Cabbage seedlings were grown in a controlled glasshouse and 10 seedlings from each genotypes were planted in 7 lt plastic pots. Plant nutrition components were applied and plants were protected with fungicides and insecticides regularly throughout the cultivation.

Carmine staining technique was used for determining the uni-nucleate microspore stage. Flower buds (3-4 mm in length and 1-2 mm in width) were collected in the middle to late uni-nucleate microspore stages (Figure 1a , Figure 1b). The buds were transferred to the laboratory immediately and rinsed under running cold tap water for 15 min in teapots. Subsequently, buds were immersed in 70% ethanol for 1 min then 10% commercial bleach solution for 15 min. Buds were rinsed three times with sterile distilled water for 5 min each time and placed on sterilized filter paper to desiccate excessive surface water. MS and B5 medium supplemented by various PGR's were used for callogenesis, embryogenesis and plantlet initiation (Table 1). The pH of the media was adjusted to 5.8 and solidified with 7 g/l agar-agar for all culture processes.

Callus induction, callus maturation, embryogenesis and plantlet initiation

1.0 mg/l 2,4-D was used with the addition of 100 g/l sucrose for both MS and B5 medium for callus induction. Anthers without filaments were excised from buds and cultured in jars containing 25 ml of induction medium. Jars were sealed with stretch film and incubated in a growth chamber at 26±1°C and illuminated with white fluorescent 32 W lamps (3000 lux) under 16/8 h (day/night) photoperiod. Five anthers for each jar and ten jars for each replicate were cultured for each medium and genotype.

When calli enlarged (Figure 1c, Figure 1d) they were cut into pieces (about 5 x 5 mm) and sub-cultured onto maturation and embryogenesis media supplemented by a combination of 30 and 100 g/l sucrose + 1.0 mg/l NAA + 800 mg/l Glutamine + 10 mg/l Serine, 2.0 mg/l Silver nitrate and 1.0 mg/l BAP at same conditions.

Five weeks old maturated calli were transferred fresh differentiation media (Figure 1e) and were incubated at same conditions. The differentiation media were refreshed 2 times at 2 weeks interval to maintain callus formation and stimulate plantlet initiation.

Data collection and statistical analysis

A factorial experiment based on completely randomized design with five replications was used. Five jars for each replicate were cultured for each medium, genotypes and sucrose concentration. The data were analyzed by SPSS and mean values were separated based on Duncan's multiple range test.

Results

At the end of this study, various types of callus form (whitish, greenish, yellowish) were obtained from the anthers in callus induction procedure. The anthers differentiated and turned callus form within 5 or 6 weeks of culture (Figure 1e). It is obvious that strong genotypic differences were determined for callogenesis, embryogenesis and plantlet initiation processes (Table 2). Interestingly, among the cultivars, Zencibas cv. had an only positive reaction and the others (Integro F_1 and Cabellero F_1 cv.) were found to be highly recalcitrant and they did not generate any response.

Besides, the medium was the other important factor in all processes and the highest responses were found statistically signıficant from MS medium. In respect to callogenesis, MS medium was twofold higher than B5 and the percentage of callus induction was 52% in MS and 26% in the B5 medium for Zencibas cv. However, sucrose concentration had a determinative effect on callus maturation, embryogenesis, and plantlet initiation. The mean percentage of callus maturation changed from 20.0% (by 100 g/l sucrose in B5) to 56.0% (by 30 g/l sucrose in MS) and MS medium + 30 g/l sucrose produced the favorable maturation results.

Some fragment of maturated calli turned mazerine blue within 3 or 4 weeks of embryogenesis process. Then, the primary embryos were seen in some callus within 5 or 6 weeks (Figure 1f-1k), subsequently, mini plantlets were seen on some callus after 3 or 4 weeks (Figure 1l and Figure 1m). Likewise, medium and sucrose concentration were found to be effective on the frequency of embryogenesis and plantlet initiation and the highest responses were observed by the combination of MS + 30 g/l sucrose. The mean

percentage of embryogenesis were ranged from 0% (by 100 g/l sucrose in B5) and 30% (by 30 g/l sucrose in MS) when it was determined by 7.5% (by 100 g/l sucrose in MS) and 12.5% (by 30 g/l sucrose in B5). On the other hand, the frequency of the mean number of plantlets per callus was 0.58 and 0.68 with the addition of 30 g/l sucrose in MS and B5 medium, respectively. Although MS + 100 g/l sucrose gave the promising outcome, the frequency was found to be the lower than average.

Discussion

To our knowledge, this is the first report on anther culture in red cabbage. According to our preliminary results, the success of androgenesis was found to be the highly depend on genotypes, medium composition, and sucrose concentration, statistically. In view of androgenic response, the genotypic reaction was found to be a key factor in our research for all process, particularly. Hence, only open pollinated local cultivar Zencibas released androgenic response, besides, the other hybrid cultivars were found to be unresponsive. This is in line with findings of many researchers, and the differences of the genotypic reaction were observed from androgenesis in winter rape (Smykalova *et al*., 2006), in rapeseed (Custers, 2003; Weber *et al*., 2005) and in oilseed rape (Gu *et al*., 2004). It is clearly reflected that genotype is the main problem for androgenesis in *Brassica* (Zhang *et al*., 2008; Lee *et. al*., 2014).

Medium composition and sucrose concentration were effective on callus induction and embryogenesis. The MS medium combined with low sucrose concentration produced the maximum percentages of embryonic callus, besides, B5 medium and higher sucrose concentration had a negative effect on both callogenesis and embryogenesis. Medium effect on embryogenesis has also been reported for microspore embryogenesis in *B. oleracea* (Dias, 1999) and *B. campestris* (Wakui *et al*., 1994), in agreement with our findings. MS medium with BAP + 2.4-D + low sucrose (20 and 30 g/l) produced the highest embryonic response in broccoli. The anthers were lost their viability in higher sucrose concentrations (50 and 60 g/l) and embryogenesis was interrupted, comparing to B5 medium (Mousa *et al*., 2014). Similarly, the best embryogenic response was obtained from on MS medium with 20 g/l sucrose + 1 mg/l BA+0.001 mg/l NAA (Krzyzanowska *et al*., 2006). On the other hand, MS medium with 1 mg/l BA produced the maximum percentages of embryos in cabbage (Gorecka and Krzyzanowska, 2007), and BAP was the most efficient PGR for enhancing shoot multiplication and elongation in broccoli (Ravanfar *et al*., 2009)

Conclusion

Anther culture is highly recommended as an alternative method to microspore culture in embryogenesis and plantlet initiation for red cabbage in dihaploidization process. However, this technique should be improved either the production high quantity in vitro regenerants or used for a broad spectrum for future breeding efforts. Moreover, the success of this technique is highly depending on genotype, and it is the main obstruction of dihaploidization process. Thus, our further investigations will be realized on fecund lines, effective PGR combinations, and concentrations, improve the physiological condition of donors and also microspore culture for alternatively.

Acknowledgments

We gratefully acknowledge the support of The Black Sea Agricultural Research Institute.

Table 1. The composition of MS and B5 media

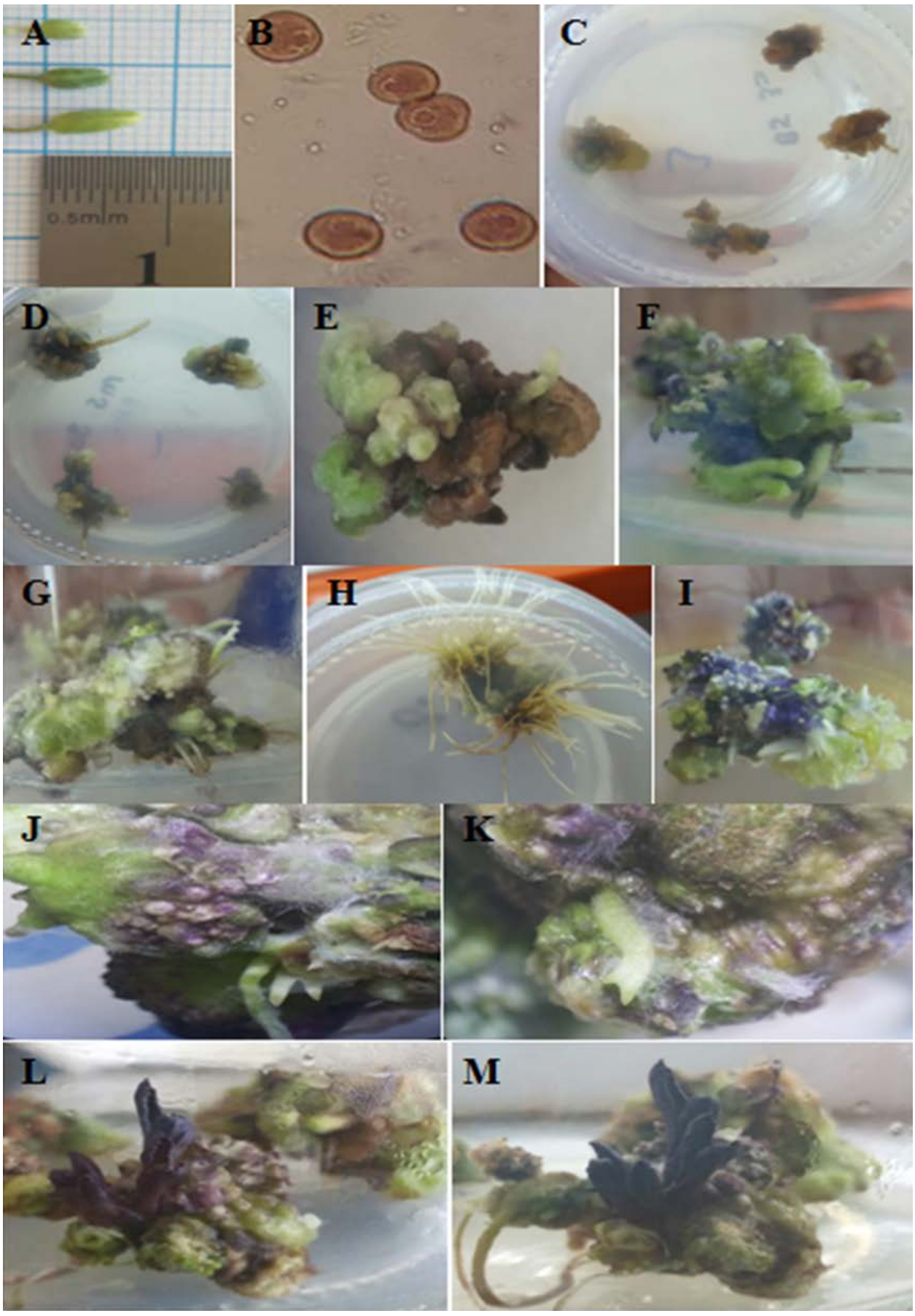
	Media	Sucrose (g/l)	2,4-D	NAA	Glutamine (mg/l)	Serine	$AgNO_3$	BAP
Callus induction	B5 MS	100	1	1	-	-	-	-
Callus maturation, embryogenesis, plantlets initiation	B5 MS	100 30	-	1	800	10	2	1

Table 2. The effects of media on callus induction (CI %), callus maturation (CM %), embryo induction (EI %) and plantlet initiation (PI plant/per callus)

Genotype	Media	CI	CM		EI		PI	
			30	100	30	100	30	100
Zencibas	MS	52.0 ^{A}	56.0 ^{A}	40.0 ^{B}	30.0 ^{A}	7.5 ^{BC}	0.58 ^{A}	0.13 ^{BC}
	B5	26.0 ^{B}	40.0 ^{B}	20.0 ^{C}	12.5 ^{B}	0.0 ^{D}	0.68 ^{A}	0.0 ^{D}
	Avr.	*39.0*	*48.0* ^{A}	*30.0* ^{B}	*21.3* ^{A}	*3.8* ^{B}	*0.63*	*0.07*

Different letters indicate a significant difference (P<0.05).

Figure 1. (A) Female flowers (B) Microspores at uni-nucleate stage (C and D) Callus induction and enlargement (E) Callus maturation (F-K) Embryogenesis (L and M) Plantlets initiation



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