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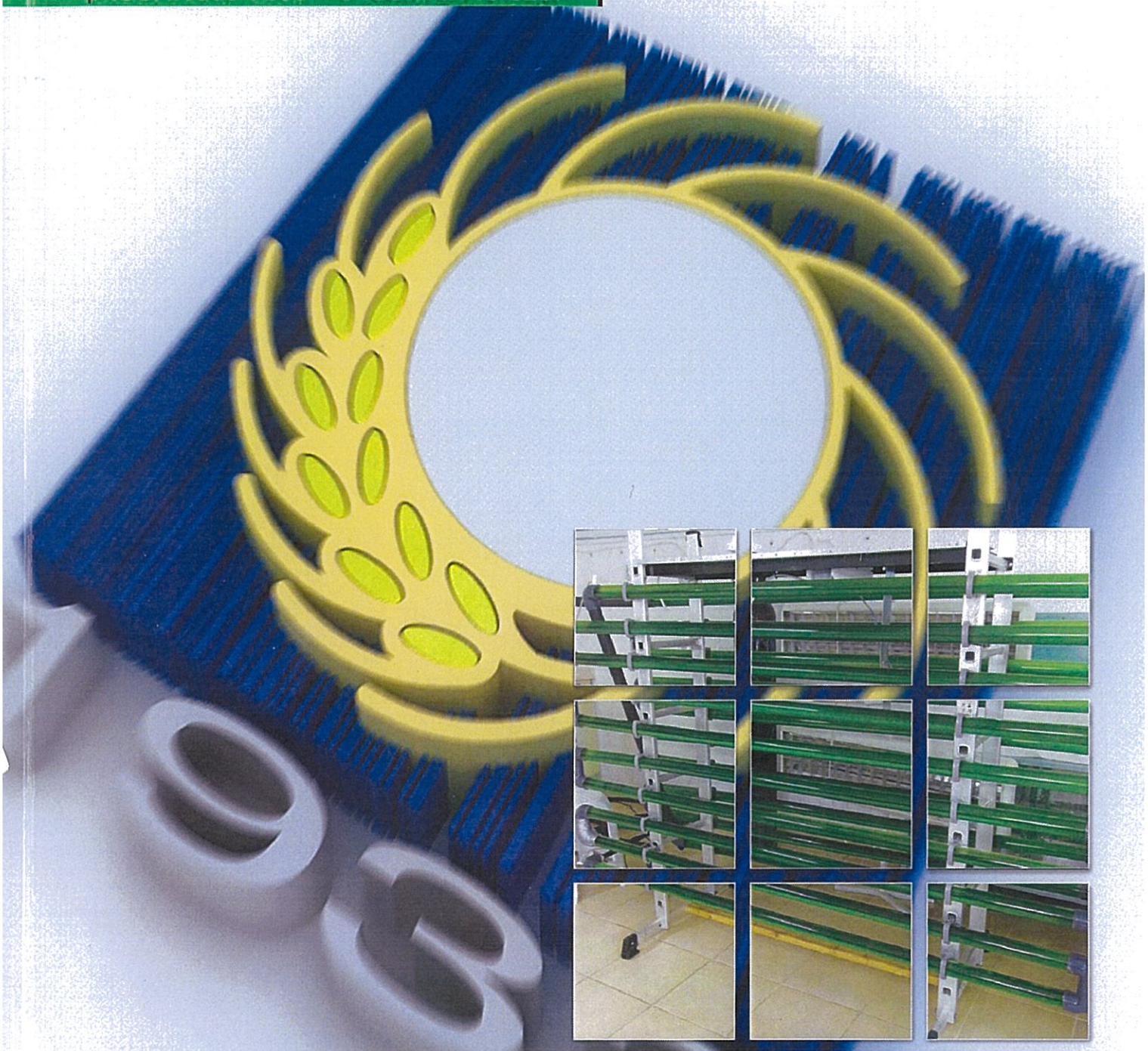
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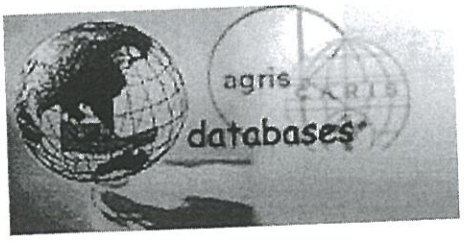
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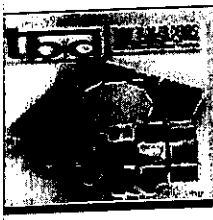
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Effect of Heat Shock Treatment on Microspore Embryogenesis in *Brassica oleracea* Species

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ABSTRACT

Heat shock treatments are widely used to induce microspore embryogenesis in *Brassica* species. In this study, the effect of high temperature treatment (32 °C and 35 °C for 2 days) on microspore embryogenesis was investigated in six genotypes of Turkish white head cabbage (Yalova-1, Ercis, 177 C, 177 T, 531 C, 538 C), three genotypes of Turkish kale (Balkaya, Yanmaz, Karadere 077) and five commercial F_1 ornamental kale hybrids (Red Piegon, Victoria Piegon, Red Chidori, white Kamome, and Pink Kamome). Microspore-derived embryos formation differed depending on genotype and high temperature. The highest embryo yield was obtained as 9.92 embryo per petri dish in cv. Yalova-1, 11.13 embryo per petri dish in Pink Kamome F_1 at 32 °C, and 5.63 embryo per petri dish in cv. Karadere 077 at 35 °C.

Keywords: Embryo; Genotype; High temperature; Microspore culture; White cabbage; Kale

Brassica oleracea Türlerinde Sıcaklık Şoku Uygulamalarının Mikrospor Embriyogenesis Üzerine Etkisi

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ÖZET

Sıcaklık şoku uygulamaları *Brassica* türlerinde mikrospor embriyogenesisi uyarmak amacıyla yaygın olarak kullanılmaktadır. Bu çalışmada, 6 Türk beyaz baş lahana (Yalova-1, Ercis, 177 C, 177 T, 531 C, 538 C), 3 Türk yaprak lahana (Karadere 077, Balkaya, Yanmaz) çeşit ve genotipi ile 5 ticari F_1 hibrid süs lahanası çeşidinde (Red Piegon, Victoria Piegon, Red Chidori, White Kamome, and Pink Kamome) yüksek sıcaklık uygulamalarının (32 °C ve 35 °C, 2 gün) mikrospor embriyogenesisi üzerine etkisi araştırılmıştır. Mikrospor kökenli embriyo oluşumu genotip ve yüksek sıcaklığa göre farklılık göstermiştir. En yüksek embriyo oluşumu 32 °C'de 9.92 embriyo petri^{-1} değeri ile Yalova-1 çeşidinden, 11.13 embriyo petri^{-1} değeri ile Pink Kamome F_1 çeşidinden ve 35 °C'de ise 5.63 embriyo petri^{-1} Karadere 077 çeşidinden elde edilmiştir.

Anahtar Kelimeler: Embriyo; Genotip; Yüksek sıcaklık; Mikrospor kültürü; Beyaz baş lahana; Yaprak lahana

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1. Introduction

Microspore culture can be utilized to shorten the duration of plant breeding programs and to obtain homozygous pure lines in *Brassica* species. Different stress treatments can be applied to flower buds, anthers or isolated microspores to stimulate microspore derived embryo formation (Shariatpanahi et al 2006; Tuncer & Yanmaz 2007; Yuan et al 2012; Cristea 2013). In all these cases, conversion to the sporophytic pathway can be induced by subjecting microspores to various stresses. The most effective and common stress treatment in *Brassica* species is the short-term heat shock. Heat shock is more effective when applied after microspore isolation. Gametophytic developmental pathway can be diverted from sporophytic pathway when optimal heat shock is applied to the isolated microspores, and microspore based embryo development increases (Ferrie & Caswel 2011).

Optimal heat shock regimes vary from species to species. For example, more successful results are noted to be attained at 32.5 °C for 1 days in broccoli (Carlos & Dias 2001; Yuan et al 2011) 32.5 °C for 1 days in *B. oleracea* var. *costata* (Dias & Correia 2002), 30 °C for 2 days (Wan et al 2011), 32.5 °C for 2 days (Kim et al 2012), 30 °C for 6 days (Ahmadi et al 2012), and 32 °C for 2 days in *B. napus* (Prem et al 2012; Wen et al 2012); 30.5 °C for 2 days in *B. oleracea* (Winarto & Teixeira da Silva 2011), 35 °C for 1 days in *B. rapa* (Zhang et al 2011), 32.5 °C for 10-15 days in *B. juncea* (Prem et al 2005) and 32 °C for 3 days in *B. carinata* (Abraha et al 2008). *Brassica* crops such as cabbage and kales widely grown in Turkey. But breeding studies on these species have progressed slightly due to cross pollination and take many years of breeding studies. Microspore culture technique is effective to accelerate the *Brassica* breeding process abroad countries. However, this technology can not to be used effectively in Turkey. In this study, the applicability of this technology has been researched in Turkish cabbage and kale genotypes/breeding lines which still continue breeding studies in Turkey.

The present study aimed to investigate effective heat shock treatment in order to promote microspore derived embryo formation by microspore culture in different cultivars of Turkish white head cabbage and kale and ornamental kale.

2. Material and Methods

2.1. Plant material and growing donor plants

Four breeding lines (177 C, 177 T, 531 C, 538 C), one variety (cv. Yalova-1) and one genotype (Ercis genotype) of Turkish white head cabbage, three varieties of Turkish kale (Balkaya cv., Yanmaz cv., and Karadere cv.) and five ornamental kale hybrids (Red Piegon F_1, Victoria Piegon F_1, Red Chidori F_1, white Kamome F_1, and Pink Kamome F_1) were used as plant material. The plants were grown in open field conditions. The seeds of white head cabbage and kale were sown in May 2011 and the seeds of ornamental kale were sown in July 2012 in peat filled multipots. The seedlings of cabbage and kale were planted in the field, while the ornamental kale seedlings were planted in pots filled with peat. The donor plants in field were watered with drip irrigation system, the plants in 15-cm plastic pots irrigated with tap water as required and fertilized as necessary with N:P:K (6:4:6) fertilizer. Cabbage heads and kale plants were harvested in October 2011 and were stored in an unheated plastic greenhouse during the winter months. Ornamental kales in the pots were kept in the unheated greenhouse during winter. Buds were harvested at the beginning of flowering. The flower buds were collected from 80 healthy plants for each genotype.

2.2. Isolation of microspores

Flower buds including at the late uninucleate stage were harvested from white head cabbage (2.5-3.5 mm length), kale and ornamental kale (4.0-4.5 mm length). A modified method of Tuncer (2010) was used for microspore isolation. The harvested buds were surface sterilized in 10% (v v^{-1}) bleach (sodium hypochlorite) with a few drops of Tween-20 for 10 minutes and then rinsed three times in distilled water (6 minutes each time). The buds (35-40 buds per

isolation) were crushed in 3.5 mL cold NLN medium (Lichter 1982) with 13% (w v^{-1}) sucrose (hormone-free, pH 6.1). They were then filtered through a 40 mm nylon mesh and collected in a glass beaker. The meshes and beakers were rinsed with 6.5 mL of the cold NLN-13 medium, and the final volume was made up to 10 mL. The resultant suspension was centrifuged at 4 °C, 900 rpm speed three times for three minutes in order to increase the microspore purity. The last pellet was re-suspended at a density of about 4×10^4 microspores mL^{-1} in cold NLN-13 liquid medium (1 mL cold NLN-13 bud^{-1}). Five mL of the suspension were cultured in a sterilized glass petri dish (6 cm diameter, 1.5 cm height).

2.3. Heat shock treatment and culture of microspores

For each treatment and replication, 5 mL aliquots of microspore suspension were dispensed into 60 mm × 15 mm sterile petri dishes (200,000 microspore $petri^{-1}$). In the heat shock experiment, isolated microspores were incubated under dark conditions at 32 °C and 35 °C for 2 days, and then maintained at 25±1 °C under dark conditions. Development stage of the embryos was observed with a binocular microscope (Leica mark, ICC50 HD model) at the end of culture period (three weeks after the isolation). petri dishes were taken on to the 60 rpm orbital shaker when globular and heart shaped embryos were visible with naked eyes and were kept shaken for 3 weeks.

2.4. Embryogenic capacity

Three weeks after the isolation, embryos were counted per petri and embryo development stages were determined as percentages.

2.5. Experimental design and statistical analysis

This experiment was designed as a factorial experiment based on a completely randomized design with 3 replications (8 petri dish was a replication, total 24 petri per treatment). The data was subjected to analysis of variance using SPSS software (ver. 13) and means were separated by Duncan's multiple range test ($P<0.05$). The results of microspore embryogenesis were quantified in terms of number of embryos produced per petri dish.

3. Results and Discussion

Heat shock treatment induced the embryo number in white head cabbage (*B. oleracea* var. *capitata* subs. *alba*) variety and breeding lines (Table 1). The difference between the temperature degrees were found to be statistically significant in all varieties and breeding lines except Ercis population ($P<0.05$) (Table 1).

The highest embryo yield at 32 °C for 2 days was obtained from Yalova-1 cultivars (9.92 embryo per petri) and 177 C breeding line (6.13 embryo per petri), while 531 C (8.29 embryo per petri), 538 C (7.13 embryo per petri) and 177 T (6.00 embryo per petri) breeding lines were more successful at 35 °C for 2 days. It could be seen that all the varieties and breeding lines except for Ercis population were temperature selective (Table 1).

Table 1- Effect of heat shock treatment on microspore embryogenesis in white head cabbage (*B. oleracea* var. *capitata* subs. *alba*)

Çizelge 1- Beyaz baş lahanada (B. oleracea var. capitata subs. alba) sıcaklık şoku uygulamalarının mikrospor embriyogenesisi üzerine etkisi

Accessions	*32 °C*	*35 °C*
	Embryos petri^{-1}	*Embryos petri^{-1}*
Yalova-1	9.92±0.71 aA*	3.96±0.55 bcdB
Ercis	3.88±0.56 cA	2.08±0.21 dA
177 C	6.13±1.13 bA	2.79±0.33 cdB
177 T	2.54±0.29 cB	6.00±1.79 abcA
531 C	0.00±0.00 dB	8.29±0.29 aA
538 C	3.21±0.61 cB	7.13±1.52 abA

*, different small letters in the same column show significant differences among the species ($P<0.05$) and different capital letters in the same line show significant differences among the temperatures ($P<0.05$)

The effect of heat shocks on microspore embryogenesis in kale (*B. oleraceae* var. *acephala*) was given in Table 2. The temperature differences were statistically significant except for 'Balkaya' variety, and 35 °C treatment was more promising in terms of microspore embryogenesis. The highest embryo yield obtained from Karadere 077 (5.63

embryo per petri) and Yanmaz (5.33 embryo per petri) varieties at 35 °C (Table 2).

Table 2- Effect of heat shock treatment on microspore embryogenesis in kale (*B. oleraceae* var. *acephala*)

Çizelge 2- Yaprak lahanada (B. oleraceae var. acephala) sıcaklık şoku uygulamalarının mikrospor embriyogenesisi üzerine etkisi

Accessions	*32 °C*	*35 °C*
	Embryos petri^{-1}	*Embryos petri^{-1}*
Balkaya	1.91±0.29 bA*	1.88±0.43 bA
Yanmaz	2.38±0.44 abB	5.33±0.72 aA
Karadere 077	3.59±0.49 aB	5.63±0.81 aA

*, different small letters in the same column show significant differences among the species (P<0.05) and different capital letters in the same line show significant differences among the temperatures (P<0.05)

In all the varieties of the ornamental kale, heat shock was found to be significant (P<0.05), except Red Chidori F_1 and Red Piegon F_1. The highest embryo number was attained from Pink Kamome F_1 variety (11.13 embryo per petri), followed by Victoria Piegon F_1 variety (8.37 embryo per petri) at 32 °C. The temperature shock at 32 °C was found to be more effective in the ornamental kale except for white Kamome F_1 variety (Table 3).

Table 3- Effect of heat shock treatment on microspore embryogenesis in ornamental kale *(B. oleraceae var. acephala)*

Çizelge 3- Süs lahanasında (B. oleraceae var. acephala) sıcaklık şoku uygulamalarının mikrospor embriyogenesisi üzerine etkisi

Accessions	*32 °C*	*35 °C*
	Embryos petri^{-1}	*Embryos petri^{-1}*
Pink Kamome F_1	11.13±2.46 aA*	4.00±0.52 abB
White Kamome F_1	1.92±0.62 dB	4.00±1.37 abA
Red Chidori F_1	4.13±1.85 cdA	5.13±2.38 aA
Victoria Piegon F_1	8.37±2.01 abA	3.16±0.11 bB
Red Piegon F_1	2.08±0.33 dA	0.79±0.40 cA

*, different small letters in the same column show significant differences among the species (P<0.05) and different capital letters in the same line show significant differences among the temperatures (P<0.05)

Globular (Figure 1a), heart-shaped (Figure 1b, 1c) and torpedo-shaped (Figure 1d, 1e) embryos were observed in the microscopic examination under microscope three weeks after the isolation. Variation of embryo development stages in varieties and genotypes with respect to heat shocks were given in Table 4. It could be seen that most of the microspores derived embryos were globular and hearth shaped, while the formation rates of torpedo embryos, which was the previous stage of cotyledon embryo, were found to be low. Torpedo shaped embryos were only observed in 538 C (12.2%) white head cabbage variety at 35 °C, in Pink Kamome F_1 (8.8%) and in Yalova-1 variety (3.3%) at 32 °C, while cotyledon shaped embryos were not observed at all the species (Table 4).

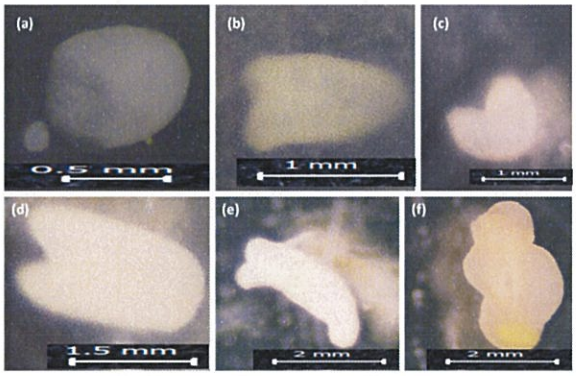


Figure 1- Embryo stages observed at 3 weeks after the isolation; a, Globular embryo (35 °C, Karadere 077); b, heart-shaped embryo (Yanmaz, 35 °C); c, heart-shaped embryo (32 °C, 177 C); d, torpedo-shaped embryo (32 °C, Yalova-1); e, torpedo-shaped embryo (35 °C, 538 C); f, embryoid (32 °C, Pink Kamome F_1)

Şekil 1- İzolasyondan 3 hafta sonraki embriyo gelişim aşamaları; a, Globular embriyo (35 °C, Karadere 077); b, Yürek şekilli embriyo (Yanmaz, 35 °C); c, Yürek şekilli embriyo (32 °C, 177 C); d, torpido şekilli embriyo (32 °C, Yalova-1); e, torpido şekilli embriyo (35 °C, 538 C); f, embriyoid (32 °C, Pink Kamome F_1)

Heat shock has been used as a trigger to induce embryogenesis in *Brassica* microspores. In *Brassica*

Table 4- The effect of heat shock on development stages of the embryos in *Brassica* species

Çizelge 4- Brassica türlerinde sıcaklık şoku uygulamalarının embriyo gelişim dönemi üzerine etkisi

Accessions	*32 °C*			*35 °C*		
	Percent of embryos			*Percent of embryos*		
	G	*H*	*T*	*G*	*H*	*T*
			White head cabbage			
Yalova-1	45.8	50.8	3.3	58.3	41.6	0.0
Ercis	100.0	0.0	0.0	100.0	0.0	0.0
177 C	53.3	46.6	0.0	100.0	0.0	0.0
177 T	38.8	61.1	0.0	44.0	55.9	0.0
531 C	0.0	0.0	0.0	25.0	75.0	0.0
538 C	36.1	63.9	0.0	27.7	60.0	12.2
			Kale			
Balkaya	100.0	0.0	0.0	100.0	0.0	0.0
Yanmaz	100.0	0.0	0.0	47.2	52.7	0.0
Karadere 077	100.0	0.0	0.0	49.9	50.0	0.0
			Ornamental kale			
Pink Kamome F_1	30.6	60.4	8.8	53.3	46.6	0.0
White Kamome F_1	100.0	0.0	0.0	100.0	0.0	0.0
Red Chidori F_1	100.0	0.0	0.0	100.0	0.0	0.0
Victoria Piegon F_1	52.0	47.9	0.0	100.0	0.0	0.0
Red Piegon F_1	100.0	0.0	0.0	100.0	0.0	0.0

G, globular-shaped embryos; H, heart-shaped embryos; T, torpedo-shaped embryos

microspores, heat shock treatments led to influence on the cell-cycle events and synthesis of heat-shock proteins (Hsp). These proteins interfere with the synthesis of gametophytic proteins while those induce synthesis of sporophytic proteins (Segui-Simarro et al 2003; Shariatpanahi et al 2006). In previous research, mostly the 30-35 °C heat shocks were suggested for different time periods (18-72 h) to stimulate microspore embryogenesis (Ferrie & Caswell 2011; Wan et al 2011; Zhang et al 2011; Kim et al 2012; Prem et al 2012; Cristea 2013). Optimal heat shock regimes differed according to the species and genotypes. In this study, these source statements were take into consideration and 32 °C and 35 °C temperature treatments were applied to microspores. Tuncer (2010) reported that treatment at 35 °C treatment is more effective in kale, and 32 °C treatment is more effective in white head cabbage (Ercis genotype), while ornamental kale cv. Red Chidori F_1 are not temperature selective for inducing microspore embryogenesis. The researcher also emphasized the requirement of repetitive studies on higher number of genotypes in order to determine the effect of 32 °C and 35 °C of temperature heat shocks clearly (Tuncer 2010). The present study aimed to examine the effects of heat shock on microspore embryogenesis more clearly by increasing the number of varieties and genotypes as suggested by Tuncer (2010). It was observed that 32 °C of temperature was more effective in cv. Yalova-1 and breeding line 177 C, while 177 T, 531 C and 538 C breeding line of white head cabbage produced more embryo at 35 °C. Although 35 °C temperature treatment was more effective in kale varieties, 32 °C temperature shock was more effective in terms of embryo stimulation for the ornamental kale.

Ferrie & Caswell (2011) reported that plant regeneration can be provided only with healthy embryos in cotyledon stage. Moreover, in some studies, some exogenous factors affecting plant

regeneration such as gibberellins, abscisic acid, antiauxin p-chlorophenoxyisobutyric acid (PCIB), 2,3,5-triiodobenzoic acid (TIBA), osmotic pressure, quality and age of embryos, embryo desiccation, and cotyledon excision, were studied to identify their influence in improving the rate of plant regeneration (Zhang et al 2006; Haddadi et al 2008; Feng et al 2009; Zhang et al 2011). In *Brassica juncea*, adding PCIB to the embryo-inducing medium not only increased the embryo yield but also played a key role in plant regeneration (Agarwal et al 2006; Zhang et al 2011). Zhang et al (2011) found that a 9.6-fold increase in plant regeneration was observed after treatment with 40 µM of PCIB. In *B. juncea*, the addition of 20 µM of PCIB led to a 5-fold increase in the frequency of microspore embryogenesis (Agarwal et al 2006).

In the present study, different rates of microspore embryo formation based on the species and varieties were obtained however plant transformation was not ensured since the embryos were not healthy cotyledonary embryos. In some cultures, embryo germination experiments could not be established due to infections, while in other cultures the infection was not occur, embryos in which liquid NLN-13 media were too small to be transferred to the solid germination media. In order to overcome sterilization problems, making different dose and duration treatments on bud sterilization in future studies can be recommended. Although studies conducted on different *Brassica* species (Agarwal et al 2006; Zhang et al 2011), it is thought to be useful addition certain antiauxin to induction medium (NLN-13) in terms of embryo maturation and plant regeneration. Genotype is the most important factor affecting the success in tissue culture techniques. Further steps might focus on foreign origin genotypes determined to be successful in microspore embryogenesis for achieving success in embryo yield and plant regeneration in vitro.

4. Conclusions

In conclusion, it was determined that effective temperature regime to stimulate microspore embryogenesis in Turkish white head cabbage, Turkish kale and ornamental kale varied depending on the species and breeding lines. These results indicate that although microspore embryogenesis was induced from microspores, it is still difficult to apply the microspore culture technique to practical breeding of *Brassica oleracea* L. genotypes with Turkish origin. Plant regeneration could not be achieved and therefore we are planning to do studies towards solving this problem in the future.

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