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CALLUS FORMATION FROM ISOLATED MICROSPORE CULTURE IN RADISH (RAPHANUS SATIVUS L.)

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CALLUS FORMATION FROM ISOLATED MICROSPORE CULTURE IN RADISH (*RAPHANUS SATIVUS* L.)

B. Tuncer

Department of Horticulture, Faculty of Agriculture, Yuzuncu Yil University, Van, Turkey
Corrresponding Author E-mail: brctuncer@gmail.com

ABSTRACT

In this study, the effects of applications of colchicine on microspore cultures were evaluated in 4 radish (*Raphanussativus* L.) varieties. For this purpose, 5 different concentrations of colchicine (0, 10, 25, 50, and 75 mg L^{-1}) were applied under dark conditions at 4°C to microspores that had been isolated in Gamborg's medium containing 13% (w/v)sucrose (B5-13). After the application of colchicine the microspores were kept for 1 day at 32.5°C in1/2 x Nitsch and Nitschliquid media supplemented with13% (w/v)sucrose (1/2 NLN-13) at a density of 40.000 microspore $mL^{-1}$and transferred for culturing under dark conditions at 25°C. Colonies of callus were observed in Petri dishes 4–5 weeks after the microspore isolation. In this study, the 50 mg L^{-1} dose of colchicine was found to be more effective in terms of callus formation. The highest callus formation was achieved from the large round radish (cv. Burkır) (6.63 calli/Petri dish)and the standard small rooted radish (cv. Cherry Belle)(5.73 calli/Petri dish) varieties.

Key words: Microspore culture, callus, colchicine, *Raphanussativus* L.

INTRODUCTION

Radish (*RaphanussativusL.*) is a *Brassicaceae* family vegetable with a wide variation and growing range, particularly in China, Japan, Korea, and Southern Asia.It is regarded as a medicinal plantthroughout the world,including Turkey. Annually, global radish production (7 million tons) accounts for approximately 2% of worldwide vegetable production (Kopta and Pokluda, 2013).The root of the radish is the most commonly consumed part, and the root sections come in various types, colors, and sizes. In addition to being rich in ascorbic acid (vitamin C), folic acid, and potassium, radishes are also good sources of B6, riboflavin, magnesium, and calcium (Zohary *et al.*, 2012).With the recentrise in people's living standards, the need for an increase in the production and quality of radishes has also increased. Therefore, the development of new varieties and the renewal of radish gene resourcesare important. However, the development of new cross-pollinated and self-incompatible radish varieties takes a long time. In species belonging to the *Brassicaceae* family, the microspore culture, which is one of the haploidy techniques can also be utilized to shorten the duration of plant breeding studies.

In previous studies, microspore culture has been applied to oilseed rape(Malik *et al.*, 2008; Takahira *et al.*, 2011), mustard(Ali *et al.*, 2008; Prem *et al.*, 2008), *B. rapa* (Zhang *et al.*, 2012; Takahashi *et al.*, 2012; Khandakar *et al.*, 2013), broccoli (Na *et al.*, 2011), head cabbage (Tuncer and Yanmaz, 2011; Yuan *et al.*, 2012; Cristea, 2013; Zeng *et al.*, 2015), ornamental kale (Zhang *et al.*, 2008), kohlrabi (Klima *et al.*, 2004), and rocket (Leskovsek *et al.*, 2008),all members of the *Cruciferae* family; however the rate of success varied according to many factors, the most prominent of which was genotype. Researchers have stated that the number of studies conducted on radisheswithin the same family is fairly limited, andan effective protocol system has yet not been developed (Chun and Na, 2011; Chun *et al.*, 2011).

Applications of colchicine with periods and concentrations varying by species to isolated microspores of species of the *Cruciferae* family are utilized to stimulate embryo formation. Colchicine applications are made at high temperatures or low temperatures. The application of 50 mg L^{-1} and 500 mg L^{-1} of colchicine under dark conditions at 30° for 15 hours to microspores isolated in *B. napus* has had a positive effect on embryo formation and quality (Zhou *et al.*, 2002).Zhang *et al.*, (2008) have performed medium renovation applications in combination with the application of 50 mg L^{-1} of colchicine for 2 days to isolated microspores of 29 different types of ornamental kale. As a result of the study, although the rate of embryo formation varied from one variety to another, it was found to be between 19.0 and 43.4 embryos/Petri dish.

Tuncer and Yanmaz (2011) reported a positive result from applications of 50 mg L^{-1} colchicine to the Yalova-1 head cabbage variety (5.3 embryos/Petri dish) and to ornamental kale (9.4 embryos/Petri dish). When colchicine was applied *in vitro* to isolated microspores in 8 genotypes of *B. napus*, the rate of dihaploidization was found to be higher than in control group (17% – 71%) for the 50 mg L^{-1} colchicine dosage (31% – 95%) for all genotypes (Weber *et al.*, 2005).The application of 10 mg L^{-1} of colchicine for a period of 1–3 days at low temperature (4°C) to microspores of 5 radish varieties

(*Raphanussativus* L.) isolated in the B5 medium increased embryo formation as compared to the control group (Bai *et al.*, 2008).

The purpose of this study, conducted on radishes for the first time in Turkey, is to investigate the effect of preliminary applications of colchicine to isolated microsporeculturesin 4 radish varieties (*Raphanussativus* L.) of Turkish origin.

MATERIALS AND METHODS

Plant material and growing donor plants: The experiment was conducted at the greenhouse and tissue culture laboratory of the Department of Horticulture, Yuzuncu Yil University, Turkey in 2015. One F_1 hybrid small- rooted radish (cv. Boncherry F_1), as well as 1 red small-rooted radish (cv. Chery Belle), 1 large-round radish (cv. Burkır), and 1 large-black radish (cv. Bursiyah) —all obtained from a private seed company— were used.The seeds were sown on vials (5.5 cm wide and 6 cm deep) containing a mixture (1:1) of peat (Plantaflor-Humus, Verkaufs-GmBH, Germany) and perlite. Seedlings that reached the stage with 3–4 leaves were planted in plastic pots(50x29 cm) containing a mixture (1:1) of peat and perlite. The sowing and planting was carried out in autumn under greenhouse conditions. Plants housed in plastic pots in a greenhouse were transferred under a low tunnel for cooling and kept in the greenhouse throughout winter. Flower buds were collected from healthy plants that reached the flowering stage.

Microspore isolation: Flower buds 2.5–3.5 mm in width, containing microspores at the late uninucleate stage of development, were collected and sterilizedin 5% commercial sodium hypochlorite solution to which a few drops of Tween-20 had been added for 20 minutes, then finally rinsed by being shaken in double distilled water 3 times, 6 minutes each time. The buds were crushed with the aid of a glass rod in the filter sterilized, cold 2 mL B5 (Gamborg *et al.*, 1968) liquid medium (13% (w/v) sucrose, pH: 5.8), releasing the microspores. The microspore suspension was subsequently passed through a metal filter (Sigma Aldrich, USA) with 40 µm pores and the residue on the filter was re-filtered in 8 mL of cold B5-13 (Gamborg *et al.*, 1968) medium and collected in 50 mL centrifuge tubes. The microspore suspension was centrifuged at 1,000 rpm at 4°C 3 times for 3 minutes each, and microspore residue was attained (Chun *et al.*, 2011).

Colchicine treatment and culture of microspores: After the final centrifugation various doses of colchicine (0, 10, 25, 50, and 75 mg L^{-1}) were applied to the microspores isolated in the cold B5-13 medium (Gamborg *et al.*, 1968) for a period of 2 days under dark conditions at 4°C. The 2% colchicine solution that had been prepared in bi-distilled water was used after being filter-sterilized. The microspores were then centrifuged twice in a cold 1/2 NLN-13 (Lichter, 1982) medium in order to remove colchicine from the medium. The isolated microspores were rendered into a suspension in the cold 1/2 NLN-13 medium, then taken into culture with 5 mL of microspore suspension (40.000 microspore / mL) in 60 x 15 mm sterile glass Petri dishes (Chun *et al.*, 2011). The hemocytometric method was used to determine microspore density. The microspores that had been taken into culture were kept for 1 day under dark conditions at 32.5°C; they were transferred to dark conditions at 25°C. When callus colonies in the Petri dishes became observable by the naked eye, the Petri dishes were transferred to a 45 rpm orbital shaker (Gerhardt Laboshake 500, Germany) and kept under dark conditions at 25°C for 3–4 weeks. Callus colonies which formed 4-5 weeks after microspore isolation were germinated in the MS medium (Murashige and Skoog, 1962)containing 3% sucrose, 0.8% agar, and 1.5 mg L^{-1}thidiazuron (TDZ), within sterile glass Petri dishes in climate-controlled rooms at 25°C for 16/8 hour light/dark cycles.

Capacity for callus formation: Colonies of callus per each Petri dish were counted 4–5 weeks after microspore isolation.

Data analysis: Each treatment was repeated 24 times. The data were subjected to analysis of variance using SPSS software (ver. 13). In order to identify different groups, Duncan's multiple range test was used ($P \leq 0.05$). The results of microspore embryogenesis were quantified in terms of number of calli produced per Petri dish.

RESULTS

The effect of colchicine treatments on callus formation varied according to variety and concentration (p□0.05). The most successful colchicine dose in all varieties was determined to be 50 mg L^{-1} (Table 1).

The highest callus formation was achieved with the 50 mg L^{-1} colchicine dose from the large-round radish (cv. Burkır) variety (6.63 calli/Petri), and the standard small-rooted radish (cv. Cherry Belle) variety (5.73 calli/Petri). The highest number of calli was also obtained from the 50 mg L^{-1} dosage in the Boncherry F1 hybrid small-root radish and the large-round black turnip (cv. Bursiyah) varieties, but the number of calli formed was found to be lower than in other varieties (Table 1). Furthermore, a statistically significant decrease (p□0.05) was noted in the number of calluses at the 75 mg L^{-1} colchicine dose in all varieties (Table 1).

The highest callus value in the control group was obtained from the standard small-rooted red radish (cv. Cherry Belle), with 2.73 calli/Petri dish. The variety with the best response to callus formation was the 'cv. Burkır'

variety in all doses, except for the control group. Figure 1 shows callus colonies observed in Petri dishes at the end of the culture period (4–5 weeks after isolation) under a binocular microscope.

Table 1. The effect of various colchicine doses in *R. sativus* cultivars on callus yield.

Colchicine dose (mg L^{-1})	Cultivars			
	Cherry Belle	Boncherry F_1	Burkır	Bursiyah
	Mean yield (callus/petri)			
0	2.73 ± 0.27^{aD}	1.10 ± 0.16^{cC}	1.76 ± 0.15^{bD}	0.56 ± 0.08^{dD}
10	3.10 ± 0.24^{bC}	1.60 ± 0.17^{cB}	3.76 ± 0.23^{aC}	0.53 ± 0.10^{dD}
25	4.00 ± 0.30^{bB}	1.46 ± 0.20^{cB}	4.60 ± 0.21^{aB}	0.90 ± 0.10^{dC}
50	5.73 ± 0.30^{bA}	2.70 ± 0.16^{cA}	6.63 ± 0.19^{aA}	2.70 ± 0.10^{cA}
75	2.00 ± 0.15^{bE}	1.23 ± 0.23^{cC}	4.36 ± 0.15^{aB}	1.76 ± 0.15^{bB}

Different capital letters in a same column show significant differences among the colchicine doses ($p \leq 0.05$). Different small letters in a same colchicine dose show significant differences among the species ($p \leq 0.05$)

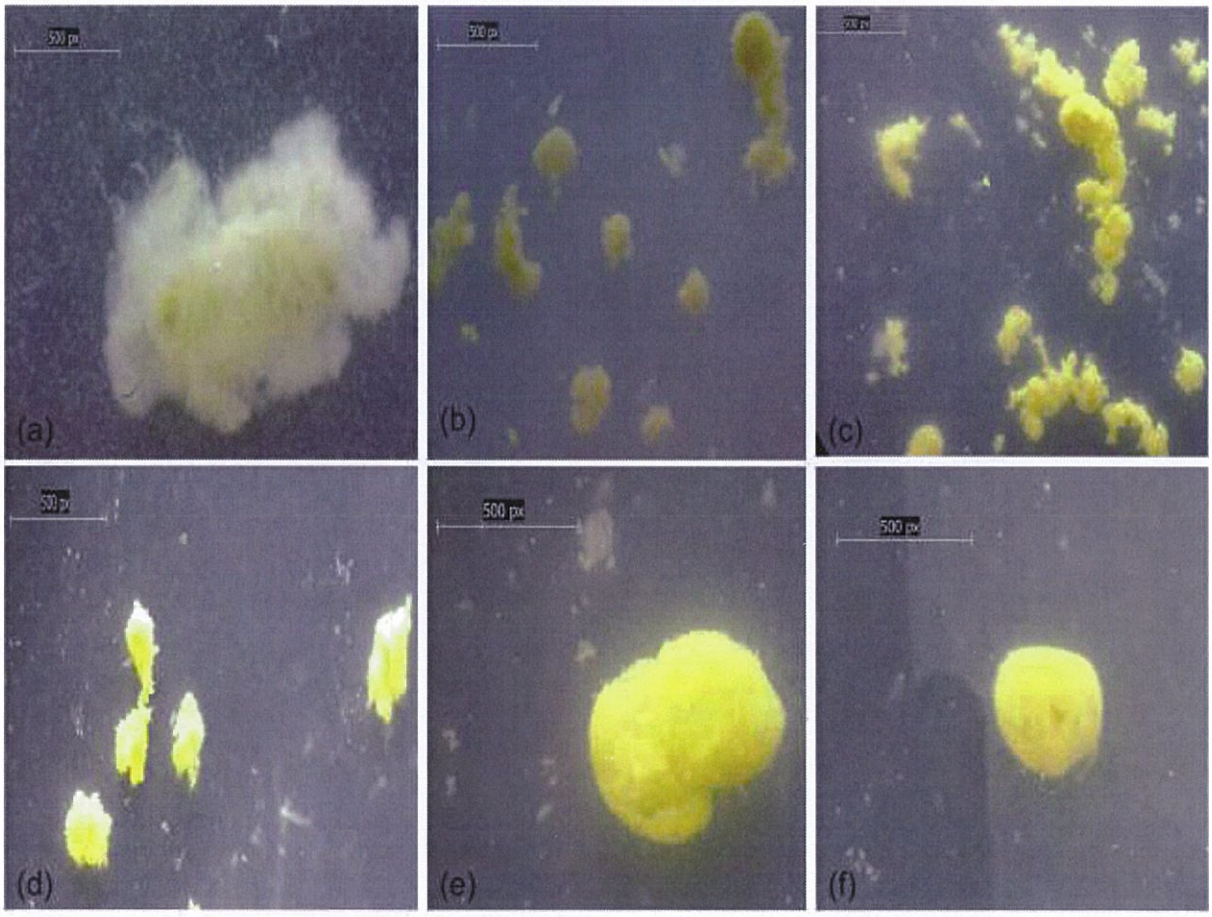


Figure 1 Callus colonies observed in Petri dishes, 4–5 weeks after the isolation: (a) 75 mg L^{-1} colchicine (cv. Burkır), (b, c, d) 50 mg L^{-1} colchicine, (cv. Burkır), (e, f) 50 mg L^{-1} colchicine, (cv. Cherry Belle).

Callus colonies that had been obtained from microspores were transferred to the solid MS medium(7 g L^{-1} agar, 20 g sucrose L^{-1}) containing 1.5 mg L^{-1}TDZand germinated in climate-controlled rooms at 25°C on16/8hour light/dark cycles. However, because since calluses that had been transferred to the germination medium had been in a very early stage, growth was not achieved and embryos could not be obtained from the calli (Figure 2).

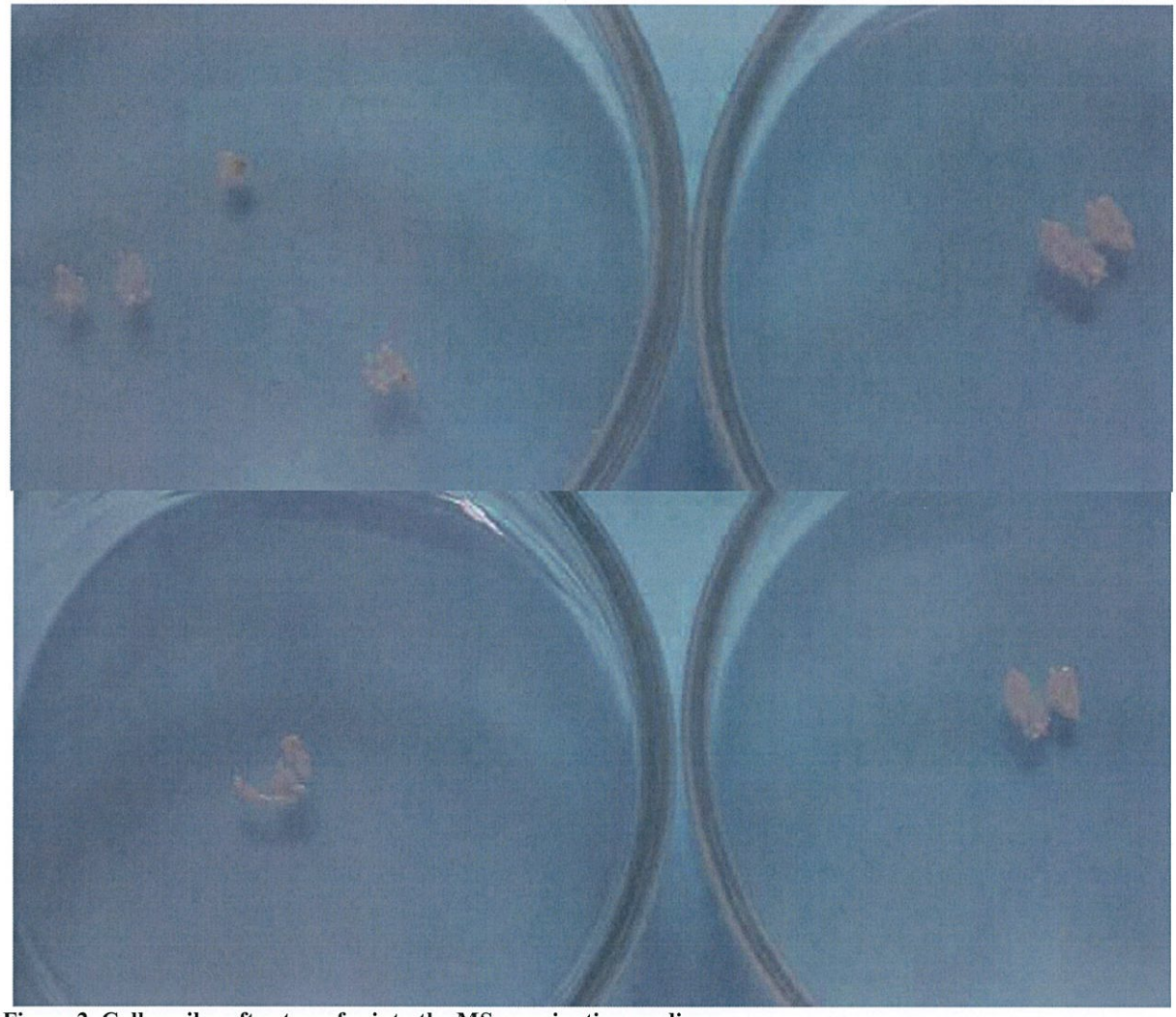


Figure 2. Callus piles after transfer into the MS germination medium.

DISCUSSION

It is reported that colchicine applications in cabbage species usually yield better results at high temperature shocks (Zhou *et al.*, 2002), whereas the same applications in radishes yield better results at low temperatures (Bai *et al.*, 2008). Therefore, considering the studies conducted on turnips, colchicine applications to isolated microspores were conducted at low temperature (dark conditions at 4°C for 2 days) in order to stimulate embryo formation. Bai *et al.* (2008) has reported that the application of 10 mg L^{-1} of colchicine for a period of 1–3 days at low temperature to turnip microspores increased embryo formation compared to the control group. However, it is also known that cold applications have a negative effect on microspore vitality, and that the isolated microspores should be taken into culture immediately. In this study, although callus colonies were obtained from microspores, the rate of success was found to be low. It is believed that this could be due mainly to the genotype effect as well as cold applications, and that therefore it could be more beneficial to make preliminary applications of colchicine on isolated microspores at temperatures of over 30°C. This is due to the fact that many research articles indicate that the stress application that is most effective in stimulating the formation of embryos from microspores of the *Cruciferae* family species is high temperature shocks.

In a study that was conducted on mustard (*Brassica juncea* L.), the formation of heart-shaped embryos was reported 10–11 days after microspore isolation—whereas embryos in the cotyledon stage were observed 18–20 days later, and embryos at the heart, torpedo—and cotyledon stage embryos were observed in

each Petri dish at 25–30 days. It is reported that of the embryos that had been transferred to the germination medium, the heart and torpedo-shaped embryos were unable to grow, and only the embryos in the cotyledon stage completed their development and turned into plants (Prem *et al.*, 2008).

With this study conducted on turnip varieties, the effects of various colchicine doses on the formation of calluses of microspore origin were investigated. As a result of this study, callus colonies were obtained from microspores, but transformation into embryos could not be achieved because growth did not occur in the calli. Notwithstanding this fact, the 50 mg L^{-1} dose of colchicine was found to be the most effective dose in facilitating callus formation from microspores in turnip varieties. In future studies conducted on microspore cultures in *R. sativus L.*, it could be beneficial to conduct studies with repetitions in intermediate doses not exceeding 50 mg L^{-1} colchicine, as well as to test colchicine applications under high temperature conditions.

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