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Full Length Research Paper

Standardization of different media for *in vitro* pollen germination of almond and evaluation of the germination capacity of stored pollen

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Almond is an important nut crop which, mostly for fruit set, needs the pollination of flowers followed by fertilization. Therefore, pollen viability and its germination capability are essential. To optimize the pollen culture medium of almond and standardization of the best medium, the present study was carried out with 48 different culture media containing different compositions, including diverse levels of boric acid (0 and 100 mg/L), calcium nitrate (0, 150 and 300 mg/l), magnesium sulphate (0 and 200 mg/l), potassium nitrate (0 and 100 mg/l), sucrose (10 and 15%) and agar (1%). Following the optimization of the best medium, pollen grains of three almond cultivars were cultured in the same optimized medium and subjected to three temperature treatments (15, 24 and 30°C) in order to determine the best thermal condition for pollen germination. Furthermore, the viability of stored pollens of these almond cultivars, three months after maintenance at 4, -20 and -80°C, was assessed through evaluation of their germination percentage using optimized medium. Maximum pollen germination (99.80%) was recorded in B2K1M2C1S2 medium containing boric acid (100 mg/l), magnesium sulphate (100 mg/l), potassium nitrate (0.0 mg/l), calcium nitrate (0.0 mg/l), sucrose (15%) and agar (1%), while the lowest (30.57%) was found in B1K1M2C3S1 medium containing boric acid (0.0 mg/l), potassium nitrate (0.0 mg/l), magnesium sulphate (100 mg/l), calcium nitrate (150 mg/l), sucrose (10%) and agar (1%). Pollen culture at 15 and 24°C showed better germination percentage than at 30°C. The data recorded for the pollen germination of stored pollens shows that Rabie pollen stored at -80°C has the highest germination rate (90.66%), while Touno pollen stored at 4°C has the lowest germination rate (36.66%). However, the findings of the present study may be of help to fruit breeders and anyone involved in pollen analysis studies.

Key words: *In vitro*, pollen viability, almond.

INTRODUCTION

Most commercial cultivars of almond are self-incompatible and to produce commercial yield (seed or kernel), pollination of flowers by fertilization is needed. For this reason, it is critical to select pollinizer(s) with high viability and germination capability in plantation and breeding programs (Kester and Gradziel , 1996; Martines-Gomes et al., 2002). The biological review indicated that the pollen grains in the special environments have the good growth and germination (Boavida and McCormick, 2007).

On the other hand, the basic components of pollen culture are calcium, boric acid, magnesium, potassium and sucrose. Generally, compounds in the pollen medium are found at different concentrations (Linskens, 1964). In addition, pH and temperature of the growth medium are two important factors that significantly affect germination and growth (Boavida and McCormick, 2007; Chebli and Geitmann, 2007). Among the elements that play a primary role in pollen culture, boron (B) has a considerable task in the development of pollen, in that it is a proposed structure prerequisite in the development of the pollen's cell walls (Matoh et al., 1996; Fleischer et al., 1998; Chene et al., 1998). Moreover, the importance of

boron during *in vitro* and *in vivo* pollen germination studies had already been pointed out (Nyomora et al., 2000; Jayaprakash and Saria, 2001; Wang et al., 2003). Boron application for germination of pollen grains is considered as an effective strategy in fruit trees (Hanson, 1991; Picchioni and Weinbaum, 1995; Nyomora et al., 1997; Nyomora et al., 1999; Hanson et al., 1985), in that application of boron on almond trees (Nyomora et al., 2000) and pear trees (Wojcik and Wojcik, 2003) has resulted to an increase in pollen germination and pollen tube growth. However, the role of calcium in pollen tube growth in recent years has also been reported (Malho et al., 1994; Malho and Trewevas, 1996; Malho et al., 2000). Pollen viability and germination capability of commercial almond cultivars *in vitro* showed that the best germination results were found in the medium having a temperature of 15 to 24°C, 10% sucrose, 100 ppm of H_3BO_3 and 2% agar (Kester and Gradziel, 1996; Martines-Gomes et al., 2002). On the other hand, the necessary processes for fruit set, pollen production, pollen germination and pollen tube growth are sensitive to high (Iwahori and Takahashi, 1964; Iwahori, 1965; Abdalla and Verkerk, 1968; Herrero and Johnson, 1980) and low temperatures (Weinbaum et al., 1984). For storage of the pollen, it is very essential to preserve its viability in suitable condition in order to eliminate the problem that may arise in time and place of artificial pollination (Khosh-khui et al., 1976). Preserving the ability of pollen germination depends on the storage conditions like humidity, temperature and air pressure (Linskens, 1964; Ranhawa et al., 1962; Snope and Ellison, 1963). Pollen viability is determined by different methods including culture on a drop through su-crose solution (2.5 to 20%) (Amma and Kulkarni, 1979), staining (Ganeshan and Alexander, 1991; Alexander, 1996), *in vitro* method and so on (Stanley and Linskens, 1974). However, concentration of 10% sucrose, 5% agar and 10ppm boric acid at 20°C was reported by Stanley and Linskens (1974) as an effective medium for germi-nation and pollen tube growth. It was found that pollen germination in culture media containing sucrose, boric acid, calcium nitrate and calcium plays an important role (Brewbacker and Kwack, 1963) in pollen germination, despite the fact that the different effects of various culture media on pollen germination of some cultivars and species have been reported (Mehan and Malik, 1975; Brewbacker and Kwack, 1963; Khan and Perveen, 2006a).

Germination capacity of stored plants' pollens has also been studied by maintaining pollens in different temperatures (Stanley and Linskens, 1974; Amma and Kulkarni, 1979; Pinney and Polito, 1990; Martinez-Gomez et al., 2001; Aslantus and Pirlak, 2002; Khan and Perveen, 2006a; Khan and Perveen, 2006b), where pollens stored at low temperature presented better germination capacity than those stored at high temperature.

Fundamentally, the objective of the present study was to optimize the pollen culture medium and the viability pollen grains of some almond cultivars following maintenance under variable temperatures.

MATERIALS AND METHODS

Branches with unopened flowers were pruned off from trees of three Almond cultivars (Rabie, Ferragnes and Tuono) growing in commercial orchards of KamalShahr, Karaj. Pollens were collected in large quantities from these cuttings for 24 h, following pruning from freshly opened blossoms. To optimize the pollen medium of almond and the standardization of the best medium, 48 different culture media containing different compositions were prepared, including diverse levels of boric acid (0 and 100 mg/L), calcium nitrate (0, 150 and 300 mg/L), magnesium sulphate (0 and 200 mg/L), potassium nitrate (0 and 100 mg/l), sucrose (10 and 15%) and agar (1%). The experiment was laid out as complete randomized design to include three replications.

Following optimization of the best medium, pollen grains of three almond cultivars were cultured in the same optimized medium and subjected to three temperature treatments (15, 24 and 30°C) in order to determine the best thermal condition for pollen germination. Furthermore, the viability of stored pollens of these almond cultivars, three months after maintenance at 4, -20 and -80°C was assessed through evaluation of their germination percentage using the optimized medium. Light microscopy was carried out under Nikon type-2 microscope. Pollen grains which produced a tube equal to their own diameter were counted as germinated. In other words, pollen tubes which were, at least, twice the diameter of pollen grains were counted as germinated, while burst pollens were counted as non-germinated (Imani et al., 2011). Germination percentage was determined by dividing the number of germinated pollen grains per field of view by the total number of pollen grains per field of view. The statistical analysis was performed using Microsoft Excel (2007) and statistical analysis software [SAS software (SAS Institute Inc, 1990], and the means were compared using Duncan's Multiple Range Test (DMRT).

RESULTS

In vitro pollen germination of three almond cultivars in various media is shown in Table 1. It is clear that the maximum pollen germination (99.70%) for Rabie almond cultivar was recorded in B2K1M2C1S2 medium containing boric acid (100 mg/l), magnesium sulphate (200 mg/l), potassium nitrate (0.0 mg/l), calcium nitrate (0.0 mg/l), sucrose (15%) and agar (1%), while the lowest germination percentage (33.00%) was found in B1K-1M2C3S1 medium containing boric acid (0.0 mg/l), potassium nitrate (0.0 mg/l), magnesium sulphate (100 mg/l), calcium nitrate (150 mg/l), sucrose (10%) and agar (1%) for this cultivar. However, pollen germination for the two other almond cultivars (Ferragnes and Tuono) was almost similar at the aforementioned media; although, considerable differences among cultivars in the ability to germinate and pollen tube growth were not observed.

The pollen viability of the three studied cultivars was found to be significantly affected during storage in variable temperatures (+4, -20 and -80° C), where the maximum germination was recorded in Rabie pollen stored at -80°C (88.56%) and the lowest (63. 44%) in

Table 1. Means comparisons of different culture media on pollen germination of 3 almond cultivars (Ferragnes, Rabie and Tuono).

Medium culture	Rabie	Medium culture	Ferragnes	Medium culture	Tuono
B2K1M2C1S2**	99.70^{a}*	B2K1M2C1S2	99.80^{a}	$B_2K_1M_2C_1S_2$	99.60^{a}
B2K1M2C3S2	98.30^{ab}	B2K1M2C3S2	99.30^{a}	$B_2K_1M_1C_2S_2$	98.07^{ab}
B2K1M1C2S2	98.00^{ab}	B2K2M1C2S2	98.00^{ab}	$B_2K_2M_2C_2S_2$	96.70^{abc}
B2K2M1C1S2	98.00^{ab}	B1K1M2C2S2	96.70^{ab}	$B_2K_2M_1C_2S_2$	96.67^{abc}
B2K1M2C2S1	96.70^{ab}	B2K2M2C2S2	93.30^{abc}	$B_1K_2M_1C_2S_2$	96.65^{abc}
B2K2M2C2S2	96.70^{ab}	B2K2M1C1S2	93.30^{abc}	$B_2K_2M_1C_1S_2$	95.30^{abcd}
B2K1M1C3S1	96.00^{ab}	B1K2M1C3S2	91.70^{abcd}	$B_1K_1M_2C_2S_2$	95.00^{abcd}
B2K2M2C3S1	94.70^{ab}	B2K2M2C1S2	91.70^{abcde}	$B_2K_2M_1C_2S_1$	91. 17^{abcde}
B2K1M2C3S1	91.70^{ab}	B2K2M2C2S1	88.30^{abcdef}	$B_2K_2M_1C_1S_1$	91.17^{abcde}
B2K2M1C2S2	91.70^{abc}	B2K1M1C2S2	86.70^{abcdef}	$B_2K_1M_2C_3S_2$	91.17^{abcde}
B1K1M2C2S2	90.00^{abc}	B1K1M1C1S2	86.70^{abcdefg}	$B_2K_2M_2C_3S_1$	91.17^{abcde}
B2K2M1C1S1	90.00^{abc}	B2K2M1C2S1	85.00^{abcdefg}	$B_2K_2M_2C_2S_1$	91.17^{abcde}
B2K1M1C2S2	90.00^{abc}	B1K2M1C2S2	85.00^{abcdefg}	$B_1K_1M_1C_2S_2$	91.17^{abcde}
B2K1M2C2S2	90.00^{abc}	B2K1M2C3S1	85.00^{abcdefg}	$B_2K_1M_2C_2S_2$	90.00^{bdcef}
B1K2M1C2S2	88.30^{bcd}	B2K1M2C2S2	83.30^{abcdefgh}	$B_2K_2M_2C_1S_2$	88.30^{cdefg}
B2K2M2C3S2	88.30^{bcd}	B1K1M1C2S2	83.30^{abcdefgh}	$B_2K_1M_1C_3S_2$	86.67^{defgh}
B2K2M2C1S2	88.30^{cde}	B1K1M2C2S1	83.30^{abcdefgh}	$B_2K_1M_1C_2S_2$	86.67^{defgh}
B1K2M1C3S2	88.30^{cde}	B2K1M1C2S1	81.70^{bcdefghi}	$B_2K_1M_2C_3S_1$	83.33^{efghi}
B1K1M1C1S2	81.70^{cde}	B2K2M1C1S1	78.30^{cdefghij}	$B_2K_1M_2C_2S_1$	83. 33^{efghi}
B1K2M1C3S1	81.70^{cde}	B1K1M2C1S1	76.70^{cdefghijk}	$B_1K_1M_1C_3S_2$	81.18^{fghij}
B1K1M2C1S1	78.30^{def}	B2K1M2C2S1	76.70^{cdefghijk}	$B_1K_1M_1C_1S_2$	81.18^{fghij}
B2K2M1C2S1	78.30^{def}	B2K2M2C3S1	76.70^{cdefghijk}	$B_2K_2M_2C_3S_2$	80.00^{ghijk}
B2K1M1C2S1	78.30^{def}	B1K2M2C2S2	76.70^{cdefghijk}	$B_1K_2M_1C_1S_2$	80.00^{ghijk}
B1K2M2C1S2	78.30^{def}	B1K2M2C2S1	76.70^{cdefghijk}	$B_1K_2M_2C_2S_2$	80.00^{ghijk}
B1K2M1C1S2	78.30^{def}	B1K1M2C1S2	76.70^{cdefghijk}	$B_2K_2M_1C_1S_2$	78.30^{hijl}
B1K2M2C3S2	78.30^{def}	B1K2M1C3S1	75.00^{defghijk}	$B_2K_1M_1C_1S_2$	75.33^{ijkl}
B1K1M2C1S2	76.70^{ef}	B2K2M2C2S1	73. 30^{efghijkl}	$B_1K_2M_2C_1S_2$	73.32^{jklm}
B1K2M2C2S2	76.70^{ef}	B1K2M2C1S1	73.30^{efghijkl}	$B_1K_2M_1C_3S_2$	71.70^{klm}
B1K1M1C2S2	76.70^{ef}	B1K2M2C1S2	71.70^{efghijkl}	$B_2K_2M_2C_2S_1$	68.30^{lmn}
B2K2M2C2S1	75.00^{ef}	B2K2M1C1S2	71.70^{efghijkl}	$B_1K_1M_2C_2S_1$	68.30^{lmn}
B2K2M2C2S1	75.00^{ef}	B1K1M1C3S1	70.00^{fghijklm}	$B_1K_1M_2C_1S_2$	68.30^{lmn}
B1K1M2C2S1	75.00^{ef}	B2K1M1C1S2	70.00^{fghijklm}	$B_2K_1M_1C_3S_1$	65.00^{mno}
B1K2M2C3S1	73.30^{efg}	B2K1M1C2S2	68.30^{ghijklm}	$B_2K_1M_1C_1S_1$	65.00^{mno}
B2K1M1C1S1	68.30^{fg}	B1K2M2C3S2	66.70^{hijklm}	$B_1K_1M_1C_2S_1$	61.70^{no}
B1K2M2C1S1	68.30^{fg}	B1K2M1C1S2	65.00^{ijklm}	$B_2K_1M_1C_2S_1$	58.35^{o}
B1K1M1C3S2	65.00^{g}	B1K1M1C2S1	63.30^{jklmn}	$B_2K_2M_2C_1S_1$	45.00^{p}
B1K1M2C3S2	55.00^{h}	B2K1M1C1S1	63.30^{jklmn}	$B_1K_2M_2C_2S_1$	45.00^{p}
B2K2M1C1S2	53.30^{hi}	B1K2M2C3S1	63.30^{jklmn}	$B_1K_2M_2C_1S_1$	43.32^{pq}
B1K1M1C3S1	53.30^{hi}	B1K1M1C1S1	60.00^{klmno}	$B_1K_1M_2C_1S_1$	41.35^{pqr}
B2K1M1C1S2	48.30^{hij}	B1K1M1C3S2	56.70^{lmnop}	$B_1K_2M_2C_3S_2$	41. 35^{pqr}
B1K1M1C2S1	45.00^{ij}	B1K2M2C3S1	56.70^{lmnop}	$B_1K_2M_2C_3S_1$	38.30^{pqrs}
B1K2M2C2S1	45.00^{ij}	B1K2M1C1S1	56.70^{lmnop}	$B_1K_2M_1C_3S_1$	38.30^{pqrs}
B2K2M2C1S1	45.00^{ij}	B2K1M1C3S1	54.70^{mnopq}	$B_1K_2M_1C_1S_1$	35.35^{qrst}
B1K1M1C2S1	43.30^{j}	B1K1M2C3S2	53.30^{mnopq}	$B_1K_1M_1C_2S_1$	33.14^{rst}
B1K2M1C1S1	43.30^{j}	B2K1M1C1S1	48.30^{nopq}	$B_1K_1M_1C_1S_1$	31.54st
B1K1M1C1S1	43.30^{j}	B1K1M1C2S1	46.70^{opq}	$B_1K_1M_1C_3S_1$	31.54st
B2K1M1C1S1	41.70^{j}	B2K2M2C1S1	41.70^{pq}	$B_1K_1M_2C_3S_2$	31.54st
B1K1M2C3S1	33.00^{ks}	B1K1M2C3S1	40.00^{q}	$B_1K_1M_2C_3S_1$	30.57^{u}

M, Magnesium sulphate; K, potassium nitrate; B, boric acid; C, calcium nitrate, S, sucrose; M1, 0 mg/l; K1, 0 mg/l; C1, O mg/l; B1, 0 mg/l, M2, 200 mg/l; K2, 100 mg/l; C2, 300 mg/l; B2, 100 mg/l; S, 15%. *Means followed by the similar letter(s) are not significantly different by Duncan test (P<0.05).

Table 2. Mean comparison of pollen germination of 3 almond cultivars, 3 months after maintenance at different temperatures.

Cultivar	Pollen germination (%)						
	3 months after maintenance at different temperatures (°C) in optimized medium[1]				Fresh at temperature (°C)		
	+4	-20	-80	Average	15	24	30
Rabie	70.76[a]	80. 76[a]*	88.56[a]	80.02[a]	98.35[a]	94.54[a]	80.63[b]
Tuono	64.56[a]	74.56[a]	82.66[a]	73.92[b]	94.32[a]	93.35[a]	79.00[b]
Ferragnes	55.00[b]	70.00[b]	74.00[b]	66.33[c]	91.52[a]	90.65[a]	71.25[b]
Average	63.44[a]	75.10[a]	81. 74[b]	73.42	94.73[a]	92.84[a]	76.93[b]

[1]Optimized medium: 100 mg/l boric acid, 0.0 mg/l sulphate magnesium, 0.0 mg/l potassium nitrate, 300 mg/l calcium nitrate, 15% sucrose and 1% agar at 24°C. *Means followed by the similar letter(s) are not significantly different by Duncan test (P<0.05).

Ferragnes pollen stored at +4°C (Table 2). Pollen viability exceeded 90% for all cultivars evaluated before storage, while average pollen germination percentage in Rabie, Tuono and Ferragnes cultivars was 80.02, 73.92 and 66.33%, respectively (Table 2), following storage in +4, -20 and -80°C.

Almond cultivars showed significantly different response with regard to their pollen viability following storage. According to Table 2, the mean values for pollen germination, 3 months after storage, decreased to 63.44, 75.10 and 81.74% for +4, 20 and -80°C, respectively. However, it was observed that differences in pollen germination following storage at +4, -20 and -80°C were significant, but there was no significant difference between pollen germination following storage at -20 and -80°C.

DISCUSSION

The data presented in Table 1 demonstrates that the medium optimized for the three cultivars of Rabie, Tuono and Ferragnes may be applied to pollen germination test in other cultivars of almond; however, Weinbaum et al. (1984) reported that the ability to compare germination between cultivars of different species would be different.

The pollen of Rabie and Ferragnes cultivars showed their lowest germination in most media lacking boric acid as compared to those supplemented with boric acid (Table 1). These observations are in agreement with previous researches (Chene et al., 1998; Wang et al., 2003) which concluded that for pollen viability test, some media are effective as compared to others.

The presence of calcium in the pollen culture medium with appropriate concentration plays an important role in pollen germination, but if not used with optimal concentration, inhibitory effects could arose (Brewbacker and Kwack, 1963; Mehan and Malik, 1975; Khan and Perveen, 2006a) and sometimes cause toxicity in the medium used in this study.

The three almond cultivars were significantly different with respect to their pollen viability following a three month period storage in variable temperatures (Table 2). Pollen germination was initially high, but later declined. Also, corroboratory results were already reported by Snope and Ellison (1963), Linskens (1964), Pinney and Polito (1990), Martinez-Gomez et al. (2002) and Khan and Perveen (2006a) who assessed the pollen viability in different conditions.

It is clear from our data (Table 2) that the three different temperatures (+4, -20 and -80°C) influences the pollen viability in a different way, such that lowering of the temperature tends to increase the level of viability. As a result, pollen germination of almond cultivars, three months after maintenance at +4, -20 and -80°C was determined as 63.44, 75.10 and 81.74%, respectively (Table 2).

These findings confirm the results of the germination capacity of stored pollens of *Abelmoschus esculentus* L. (Khan and Perveen, 2006a), the germination capacity of stored pollens of *Solanum melongena* L. (Khan and Perveen, 2006b), the low temperature storage of almond pollens (Martinez-Gomez et al., 2001), olive pollens storage and *in vitro* germination (Pinney and Polito, 1990). However, the increasing germination capacity of strawberry pollens in low temperature observed in Aslantus and Pirlak's (2002) study also concur with those of Stanley and Linskens (1974) and Amma and Kulkarni (1979), where pollens stored at low temperature presented better germination capacity than those stored at high temperature. Pollens stored at low temperature, that is, at -80°C, showed better germination percentage as compared to those stored at -20 and 4°C. This condition seems to have more potential to maintain viability as compared to other conditions. Also, pollen culture at 15 and 24°C showed better germination percentage than at 30°C. Nonetheless, it is anticipated that the results of this research will be used for pollination management and hybridization programs of almond.

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