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Effect of 2,4-D and 2ip hormones on embryogenesis callus production and the effect of sucrose and concentrations of MS salts on somatic embryogenesis of date palm (cv. Medjool)

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ABSTRACT: Somatic embryogenesis is one of the most common and effectual method for in vitro regeneration of date palms. In this study, production of embryogenesis callus in Medjool cultivar on high concentrations of 2,4-D (10, 30, 50, and 70 mg/l) with 2ip (0 and 3 mg/l) were examined. In the absence of 2ip hormone and the treatment with 70mg/l 2,4-D hormone, we witnessed the highest embryogenesis callus production of (2.68g) and production of embryogenesis calluses of about 2.57g when we used 3mg 2ip hormone with 50mg 2,4-D, accounted as the second order of production. Moreover, as we aimed at producing somatic embryos, embryogenesis calluses were exposed to two concentrations of 30 and 60mg/l sucrose and three different concentrations of MS salts, by which it was determined that the higher concentration of sucrose was applied, the more the number of embryos were produced. It was also revealed that for producing somatic embryos, 1/2MS and 3/4MS were more efficient than absolute MS.

***Keywords*:** Date Palm, Embryogenesis Callus, Somatic Embryogenesis, 2,4-D, and Sucrose

INTRODUCTION

Being out-crossed, perennial, and monocotyledon, date palms (*Phoenix dactylifera L*.) are very heterozygote. This plant belongs to *Areaceae* genus, well-known for its ability to tolerate high temperatures. In the recent years, date palms have been importantly considered as a traditional crop in 40 countries such as USA, South Africa and Australia. Throughout the world, date palm production has been increasing in the 3 past decades (FAO, 2008). By producing date palms over 1,200,000 tons per year, Iran ranks first in date production in the world.

In order to pace up date palm breeding programs, overcome the traditional problems and meeting the demands, it is necessity to improve the propagation method of date palm by means of tissue culture techniques (Mujib ., 2004; Eshraghi ., 2005). Tissue culture is generally a technique by which propagation of fruit trees like date palms can be practically achievable.

In vitro propagation of date palms is performed via/through two different methods; direct organogenesis and somatic embryogenesis, based on callus taken from meristem. Somatic embryogenesis is a successful method that has been commonly used worldwide in order to produce date palms. Being commonly practiced as a successful technique worldwide, somatic embryogenesis is used for mass propagation as well (Kunert . 2003). As far as the acceptability and feasibility of the method is concerned, it is proved to be suitable for micropropagation in large scale commercial purposes (smith . 1995). Somatic embryogenesis, which differentiates the embryos from somatic cells,

not oocyte, has been widely used as it has too many advantages (Carlos and Martinez 1998). In this method, instead of forming just one organ from the explant, a mass of undifferentiated cells called callus is produced. This method goes through some stages like callus induction, embryogenesis callus production, embryo development and maturity, germination, and rooting.

Propagation of embryogenesis calluses takes place in an MS media which is supplemented by 2.25uM 2,4-D, 0.83uM kinetin, and 2uM ABA (Huong. 1999). The great number of embryos was produced in a culture containing NAA (0.1 mg/l) and 2ip (0.2mg/l) (Gaber., 2010). The most reliable media for inducing somatic embryos are those that are supplemented by NAA (0.05 mg/l) and 2ip (1 mg/l) (Al-Baiz ., 2000). The use of 1mg/l of 2,4-D increased the production of somatic embryos (El-Bellaj, 2005). Nakagawa ., 2001 reported that it is effectual in production of somatic embryos in the media. Growing the developed somatic embryos on half of MS media, enriched with 60g/l sucrose and 2mg/l ABA, led to production of thicker and taller embryos with high concentration of protein (Fki, 2005). Since both stages of production of embryogenesis calluses and formation of embryos are the most important stages of somatic embryogenesis and also due their significance in this research, finding an optimum media for these two stages is of major interest.

MATERIALS AND METHODS

This research was carried out by a non-native Iran date palm called Medjool, which is the origin of North Africa. As this cultivar produces large fruits, it was preferred over the Iranian ones.

Some 3-4-year-old shoots of Medjool cultivar were gathered during the winter and then the leaves were removed from them, what remained was the apical meristem and its early leaves. The remained parts were washed by running water for 30min and then suspended in ethanol 70% for 1min, and later they were put into sodium hypochloric 2.5% and 20m/l tween solution for 15-20 min. Finally, the samples were washed 4-5 times by sterile distilled water. After disinfection, samples were cut and then the outcome explants (including apex and 2 early leaves) were cultured on the media. In all different stages of this experiment, we used MS as a primarily media supplemented by NaH2PO4 (170 mg/l), Mayo-Inositol (125 mg/l), Glutamine (200 mg/l), Thiamine-hydrochloride (1 mg/l), nicotinic acid (1 mg/l), Pyridoxine-hydrochloride (1 mg/l), sucrose (30 g/l), and agar (7 g/l) and in each stage it was altered, as required. Before adding agar to the media, the pH was adjusted to 5.6-5.7 and then the media was autoclaved at 121°C for 20min. Then, sterilized shoot tips were disseminated on the establishment media, supplemented with 2, 4 – D (100 mg/l), 2ip (3 mg/l), and activated charcoal (3 g/l), and stored for 12 days in complete darkness at 3 ± 24 ° C, taking subculture with a 3-week interval.

In order to produce more calluses, even after passing this period, the initial calluses still remained in the same media. After calluses were made, in order to form embryogenesis callus, the explants were then transferred into 8 different media with different hormonal treatments. This research was conducted in the factorial form and in a completely randomized design with five replicates (with each replicate consisting of 3 petri). Hereby, the first factor is 2ip hormone with 0 and 3mg/l concentrations and the second one is 2,4-D hormone with a variety of concentrations of 10, 30, 50, and 70 mg/l. Specimens were, then, grown on such cultures for 12 weeks and after each 3 weeks their subculture were performed and at the end of this 12-week period the weight of produced embryogenesis calluses was measured in grams.

In the next step, aforementioned calluses were introduced to 6 media which differed from each other, with different concentration of salts like MS (MS, ½ MS , ¾ MS) and sucrose in two concentrations (30 and 60 g/l). Regarding the treatments, there were six different treatments including 2,4-D (1mg/l), 1/2MS+2,4-D(1mg/l), 2,4-D (1mg/l)+60g Sucrose, 1/2MS+2,4-D (1mg/l)+60g Sucrose, and 3/4MS+2,4-D(1mg/l). Having been remained in these cultures for 9 weeks, the samples were subcultured after each 3 weeks. This experiment was performed in a completely randomized design with 6 replicates (with each replicate comprising 3 petri). After 9 weeks, the number of those embryos which were outcome of 10g embryogenesis callus was estimated.

Variance analysis was drawn via SAS and mean comparison was executed by Duncan multidimensional method at 5%.

RESULTS AND DISCUSSION

Analysis of Variance of embryogenesis callus weight treat (Table 1) shows that after 12 weeks (four subcultures) simple and interaction effects between different hormonal treatments were significant at 5%. Effects of hormone 2ip in two different concentrations (0 mg / l, 3 mg / l) on the weight of the embryogenesis callus (in grams) in Figure 2 indicates that the absence of this hormone leads to the production of more embryogenesis callus (1.90 g) as compared with its presence at concentration of 3 mg/l (162g).

The comparison table (Figure 2) shows that using 30 and 50 mg of hormone 2,4-D resulted in producing the highest weight of embryogenesis callus about 2.34 and 2.25 g respectively, as opposed to applying the same hormone in 10mg which produced only 0.73g, the lowest embryogenesis callus. Accordingly, using high concentration of hormone 2,4-D has a critical effect on improving callus weight, however, increasing too much of it would not have great effect on embryogenesis callus weight. These results endorse those of Eshraghi , 2005 which reported in order to induce embryogenesis callus in Mordarsing cultivar, there must be a high concentration of hormone 2,4-D.

Figure 3 shows the interaction of 2,4-D and 2ip hormones on embryogenesis callus weight (in grams), implying that in the absence of hormone 2ip, using high concentrations of 2,4-D (30 and 70 mg/l) would cause appropriate results. Hereby, having a high concentration of 2,4-D hormone would play a positive role in increasing embryogenesis callus weight once it is concomitant with 3mg/l of 2ip hormone; though, embryogenesis callus weight wanes in high concentration of 2,4-D (70mg/l). As a result, in the absence of 2ip hormone and with concentration of 70mg/l 2,4-D, we witnessed the highest production of embryogenesis callus about 2.68g. In addition, employing 3mg 2ip hormone with 50mg/l 2,4-D results in producing 2.57g embryogenesis callus occupies the second level of excellence and after this 10mg/l 2,4-D hormone with producing the least weight of embryogenesis callus.

Thus, it is relieved that 2,4-D hormone alone and in high concentration about 70mg/l is the best hormone to produce embryogenesis callus. However, combination of auxin and cytokinin hormones has also a positive impact on inducing embryogenesis callus, in which there must not be a high concentration of 2,4-D hormone. These results are in contrast with those of Eshraghi , 2005, in which it was reported that the optimum media for producing embryogenesis callus in Mordarsing cultivar is an MS which is supplemented with 150g/l 2,4-D and 3mg/l 2ip.

Table1. Analysis of Variance of embryogenesis callus weight from Medjool cultivar of date palm

		Mean square
Source of variation	df	Weight of callus
Growth regulator treatment (A)	1	0.79**
Growth regulator treatment (B)	3	5.48**
AB	3	3.90**
error	32	0.11
cv	39	18.95

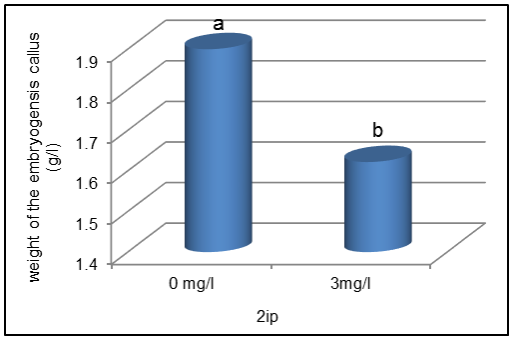

Figure 1. Effects of hormone 2ip on embryogenesis callus weight

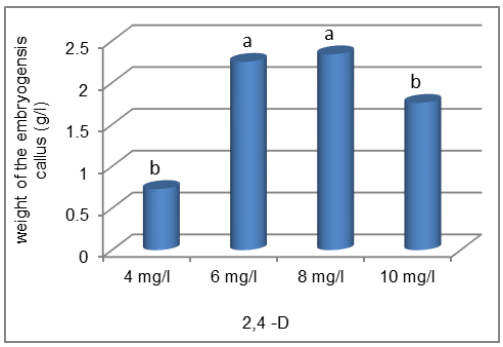

Figure 2. Effects of hormone 2,4 -D on embryogenesis callus weight

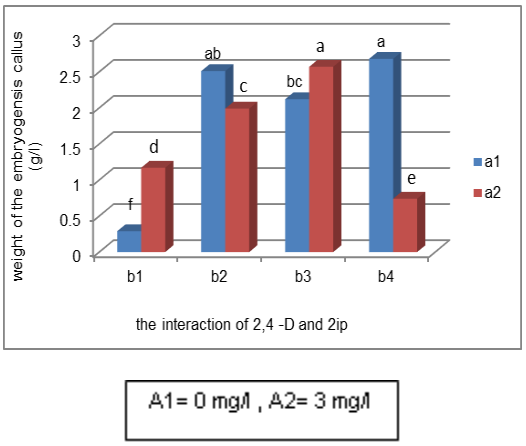

Figure 3. The interaction of 2,4-D and 2ip hormones on embryogenesis callus weight

In the next step, embryogenesis calluses were put into the media, suitable for producing and maturing the embryo. The variance analysis of number of the embryo trait (table 2) shows that different treatments were significant at 5% level. Figure 4 portrays the effect of 6 treatments after 9 weeks on producing the number of embryos from 10g of embryogenesis callus of Medjool cultivar. Among the treatments, the media that contained 60g sucrose+2,4-D(1 mgl^{-1})**+**3/4 MS resulted in producing more embryos (382.5) and the media in which there where 1/2MS+2,4-D(1mg^{-1}) was at the second grade of producing embryo by yielding 355.5 embryos per each 10g embryogenesis callus.

At last, the media with 2,4-D(1mg^{-1}) induced the least embryo production (117.5). Accordingly, it is crystal clear that sucrose has a positive influence on embryogenesis which by doubling the amount of sucrose the embryos would also be doubled. Embryogenesis induction was achieved by transferring the cells into a basic media contacting a high concentration of auxin. The most effectual auxin used for date palms was 2,4-D. Adding 1mg 2,4-D in the presence of active carbon in a low concentration (300mg/l) increased differentiation of the somatic embryos. Fki , 2003 showed that treating Deglet Nour cultivar of date palm with 1mg/l 2,4-D would increase embryogenesis from somatic cells, as an outcome. In addition, Gadalla in 2007 reported that adding 0.5 or 1 mg/l 2,4-D to Khallas cultivar resulted in increasing the number of somatic embryos. The number of embryos has a direct correlation with increasing the concentration of sucrose. These results are unanimous with the findings of Alkhateeb, 2006 which demonstrated using sucrose in high concentration (60g/l) increased production of embryos, and also Lou and Kako, 1994, reported that embryo genesis would be doubled by increasing the concentration of sucrose. It was also reported by Nakagawai , 2001 that in an effective growth culture, sucrose is contributing to constituting somatic embryos. Moreover, supplementing growth media for cucumber (Luo , 1996) and watermelon (Nakagawa ., 2001) with high concentration of sucrose increases induction of somatic embryos. Inasmuch as in tissue culture carbohydrates are used as an osmotic agent to assist tissue growth and a carbonic operative essential for lasting growth on media, altering the amount of carbohydrates would leave a considerable effect on proliferation of date palm (Zouine and Hadrami, 2004). Among all the carbohydrates, sucrose is more used in tissue culture (Iraqui and Trembly, 2001). Sucrose is absolutely essential for developing embryos and its concentration has a great influence

on embryogenesis. By increasing osmotic pressure, sucrose will trigger cell tissue groups of embryogenesis callus and thus leading to formation of somatic embryos in culture (Nakagawa ., 2001).

In this research, it is demystified that by increasing the concentration of this carbon resource embryogenesis would increase as a result. In this experiment, it was also unveiled that producing somatic embryos 1/2 MS and 3/4 MS were better than the absolute MS.

Table 2. Analysis of Variance of number of the embryo from Medjool cultivar of date palm

		Mean square
Source of variation	df	Number of embryo
Growth regulator treatments	5	64528.63
error	30	31.48
cv	35	2.12

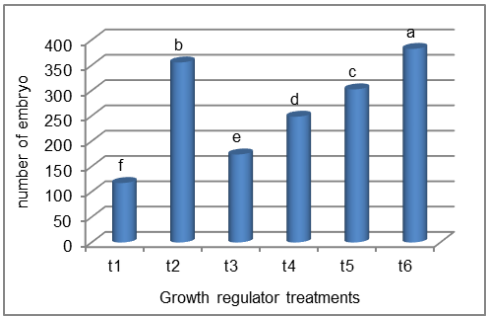

Figure 4. the effect of 6 treatments on the number of embryos

CONCLUSION

Results of this study showed that having a high concentration of 2,4-D hormone would play a positive role in increasing embryogenesis callus weight once it is concomitant with 3mg/l of 2ip hormone. it was determined that the higher concentration of sucrose was applied, the more the number of embryos were produced. It was also revealed that for producing somatic embryos, 1/2MS and 3/4MS were more efficient than absolute MS.

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