Journal of Novel Applied Sciences
Available online at www.jnasci.org
©2015 JNAS Journal-2015-4-4/445-452
ISSN 2322-5149 ©2015 JNAS

EXAMINING THE IMPACT OF PLACE, SIZE AND HARVEST TIME EXPLANTS ON CALLUS PRODUCTION OF COMMERCIAL VARIETIES OF DATES

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ABSTRACT: In order to study asexual embryogenesis of commercial varieties of Khanizi, Khasouei and Pyarom in Hormozgan province and the impact of the medium on this process, achieving embryos producing callus was inevitable. Due to cell division and slow growth of dates cell is similar to other perennial tree crops, selection of explants from the apical meristem of 3-4 year old off shoots, exploration on the impact of the apical meristem place in three commercial varieties of dates on production of callus issues is a necessity. To control pollutions on meristematic surface , a bleach solution (5.25% sodium hypochlorite) in concentration 10, 20,30,40,50, and 60 percent and for control of bacterial contamination of early detection which were minus gram and short bar shaped, the anti-biotic Ampicillin, Erythromycin, Gentamycin, Kanamycin and Tetracycline in concentrations 20,40,80 and 100mlg/l with medium were utilized. The explants were extracted from three apical meristem, In three size 2,4,and 8mm during four seasons, leaf are of each type after microscopic sections and were cultured on the Murashige and Skoog (MS)medium Skokie having 452.5 µm dichlorophenoxy acetic acid (2**,**4-D) and 14.7 µm (2-isopentenyl)adenine (2-iP) and 3g/l activated charcoal . In the present study the 40 per cent concentration of the bleach solution controlled the surface pollutions up to 94%with a significant difference compared with other concentration with the least possible burn rate. From among three antibiotics addition of Gentamycin and Kanamycin 100mg/l concentration per the medium, removed bacterial contamination about 65 to 80 per cent. The highest rate of callus production was achieved from culture of the most inner apical meristem up to 448.51mg. The varieties Khasouei, Khanizi and Pyarom showed the greatest rate of callus production after 3 months. The produced callus had a torpedoes appearance. On the other hand, the 8mm explants with average 347.96mg produced more callus compared with smaller pieces. The spring explants produced higher callus in comparison with other season's explants. The Khasouei, Khanizi and Pyarom produced the greatest amount o callus after 3 months.

***Keywords*:** Dates, Tissue Culture, Callus, Embryogenesis.

INTRODUCTION

Date is a monocot and two basic plant belongs to the *Arecaceae* family and are broadly cultivated in dry lands of East Asia and North Africa (8). Date is one of the most strategic export products of Iran (14). There are two ways for increasing dates as sexual increase with seed and asexual increase by welding (22). When the plant s increased by seeds , half of palm trees are male when they flower it could be recognized they are male which removal and replacement are very time consuming . In addition, the plants are produced via seeds are highly heterozygous (6). Through the asexual increase by welding which is one of the most common methods for production of female plants and similar to rootstock, some limitations exist. This increase method is very slow since the welding must be attached

to the rootstock for 2-3 years in order to generate a good root system (3). In addition, separation methods of body welds are very difficult and the body welds are established in soil differently (about 30-80 per cent) (7,23). Young trees create body welds (9). After the adolescence period and in puberty, lateral buds generate more inflorescences. However, any attempt for production of more body welds according to the demands through chemical and physical manipulations has been unsuccessful (28). On the other hand, some of varieties do not produce body weld at all. Moreover, fatal diseases like Fusarium wilt has been a cause of death for many date varieties. The case is a soil-borne fungus, Fusarium Oxysporum Sp (1). The selection and proliferation of new genetic samples with high adaptability to the warm weather and resistance to this disease is necessary. So, the welding body proliferation method does not meet the high demand and proliferation of pure lines through seed is impossible since dates is bipod (13,14). Therefore, micropropagation is an acceptable strategy for mass proliferation of commercial varieties of dates (7). Although, the tissue culture technique is utilized for proliferation of many varieties , but its success in cultivating wooden plants in general (10) and monocot plants for specific (2,29). According to the above points and since a significant part of the nurseries have been destroyed by the war and reconstruction of nurseries are among the priorities of Iranian gardening programs, knowing about reaction of different varieties of dates towards extraction of micro propagation in production of callus tissue, rapid proliferation and increasing yield and quality of the commercial varieties of Hormozgan province. It should be mentioned here that at few countries have at present the dates proliferation technology through asexual embryogenesis and achieving this technology is very difficult.

MATERIALS AND METHODS

Two and three year old body welding weighting 5 to 7 kg was prepared from Khasouei, Khanizi and Pyarom during four seasons from productive mother trees of Minab city. They were put into wet cotton sack and transferred to the nursery of Agriculture Department of Hormozgan province. Then, in the tissue culture laboratory via isolation of older leaves. 2,4 and 8mm meristems tissues were extracted. They were put into soluble antioxidants containing 100mgl citric acid and 150mgl ascorbic acid to be used for culturing. The bleach solution containing sodium hypochlorite 5.25% with 40% concentration for 15min with 3 drops Tween20 per 100ml soluble. Then, during four times, the tissues were washed with distilled water so the surface of the sample was cleaned from Clorox solution. Immediately, the samples were put inside the sterile antioxidants solution for getting prepared to be cultured. To control bacterial contamination, verities of antibiotics were used based on the Johnson and Case (1982) method. A layer of sterile agar containing 5% glucose was vaccinated by the infected samples. The first part of the infected Petri became infected and then similarly, the next section was infected. The Petries were kept in a reverses situation at 35 ° C for 48 h until separation stage. After that, the resulting colonies were isolated and cultured. First of Petri were marked, and then a drop of sterile distilled water was placed in the center of the Petri. A small portion was removed from each colony and was suspended in distilled water. Then, the suspensions were placed into separate circles (Preparation for drying). Drying was done via crossing 2-3 times alcohol light. For coloring, dry layer was covered by crystal violet and was kept in this situation for 30 seconds. Then they were rinsed thoroughly with sterile distilled water. Before the samples are dried, for 30 seconds they were covered with teeth and were washed with 95 per cent colorless and with distilled water alcohol. In this stage, the color was added for 30 seconds and then rinsed in sterilized distilled water and was dried with a paper towel and were dipped in oil .two Hinton agar plates for each colony was extracted and marked. Each culture was placed in sterile conditions inside three plates. The plates were tapped gently to get better contact with the agar. The antibiotics discs of Ampicillin, Erythromycin, Gentamycin, Kanamycin, and Tetracycline were used. For sterilization all glasses and instruments were placed at 121 ° C and pressure 06 / 1 kilogram per square centimeter autoclave. Duration of exposure in an autoclave varied in based on the container size and type of material placed in an autoclave, ranged from 20 to 35 minutes . In this experiment, MS medium with 170 mg/l NaH2PO4.2H2O, 125 mg/ l- inositol , 200 mg/ l glutamine , 1 mg/l pyridoxine, 1 mg/l biotin,1 mg/l pantothenate calcium, 2 mg/l glycine , 8 g/l agar , 4 g/l activated charcoal and 30 g/l sucrose was used as the basal medium. The callus induction medium was used based on the medium recommended by Alkhatib (2006), which also was used by investigators like Tahah et al (2001), Alkheiri (2001), Alkhatib and Ali Dinar (2002) , and Alkheiri and Albohrani (2004) was prepared with slight modifications. For callus induction 100 mg/ l- 2, 4 - Dichlorophenoxy Acetic Acid and 3 mg/l 2iP, and 0.5 mg/l vitamin D3 were added. The culture medium was constant at all stages of tests and treatments.

The effect of variety and location of taking explants on the amount of producing callus tissue

In this experiment, the apical meristem of three date palm as Khanizi, Khasouei and Pyarom were prepared from A, B and C parts (Fig. 1). The samples were grown in callus culture medium for callus induction. The cultures were maintained in absolute darkness and temperatures of 2 ± 25 ° C. The samples were re-cultured every 3 weeks. After 3 months of growth, fresh callus were weighted and the amount their wet weight were recorded.

Controlling Phenolic substances in the medium

One of the most important dates meristem culture is phenolic substances produced when are exposed to the air. To reduce the production of phenolic substances, antioxidants ascorbic acid and citric acid were used to preserve the sample during preparation and culturing explants and in combination with the culture medium. These antioxidants with activated charcoal were used to control phenolic substances.

Also keeping cultured meristems in conditions of absolute darkness, was another treatment for the controlling the production of phenolic substances (15).

The data analysis

The experiment was a factorial experiment in a completely randomized design with three replicates and each replicate included three observations. In this experiment, the effect of location and type of explants in 3 levels were considered. The SPSS software was applied to analyze the data and to draw diagrams the Excel software was used and the Tukey-test for comparison of the mean scores (20).

RESULTS AND DISCUSSION

DISCUSSION AND CONCLUSION

Surface Antiseptic

In order to control surface contamination and ensure the absence of bacterial and fungal agents, after the explants were treated with a bleach solution (Containing sodium hypochlorite 5.25%) at different concentrations, rate of fungal or bacterial contamination were evaluated after two weeks . the test showed that for surface antiseptic of explants, 40% bleach concentration is the best concentration. At this concentration, negligible contamination is 6%. But at the same concentration, the plant burns about 13 %. At concentration 50 and 60 percent no surface contamination was observed. But more than 85 percent the explants had burns over 30%. The results show that the concentration of 40% sodium hypochlorite for 15 minutes is the best concentration for surface antiseptic of the dates explants. Flame was used for the surface antiseptic of terminal shoots of dates controlled over 80 per cent of the surface contamination treatment. That can effectively replace with the bleach solution because of higher costs and being more time consuming. Also, the possibility of transmission of infection from the infected explants is greater than normal explants.

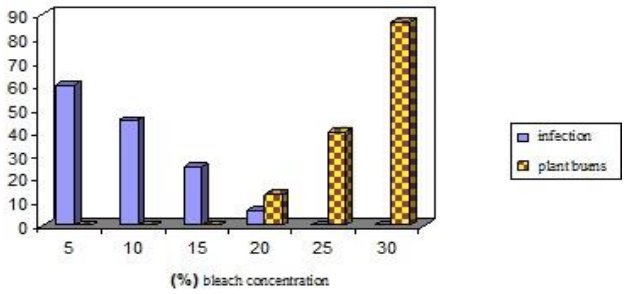

Figure 1. the impact of different bleach concentration on rate of infection and plant burns

Interior Antiseptic

Bacterial infections among 80 % of three varieties explants was observed after surface Antiseptic For detection of contamination, the explants section in contact with the medium of infection agent was isolated and stained. In this study the infection agent was of Gram-negative bacteria and was diagnosed with a form of short rods. In order to determine the susceptibility of bacteria to antibiotics a test was used by antibiotic disc. The results showed that the bacteria are sensitive to antibiotics Gentamycin and Kanamycin. Increasing concentrations of Gentamycin and Kanamycin to 100 mg/l of culture medium leads to controlling bacterial contamination approximately 65-80% (Table 1).

Table 1. the impact of antibiotic discs on prevention from growth of bacterial isolated from apical meristem of cultured dates

Antibiotics	Disc power (mc/g)	Inhibition zone diameter (mm)			
		Isolation 1	Resistance	Isolation 2	Resistance
Ampicillin	10	0	Resistant	0	Resistant
Erythromycin	10	0	Resistant	0	Resistant
Gentamycin	10	15	Sensitive	16	Sensitive
Kanamycin	30	20	Sensitive	20	Sensitive
Tetracyclin	30	16	Semi-resistant	18	Semi-resistant

Test of callus induction from meristematic explants

To achieve the different varieties Khanizi, Khasouei and Pyarom, examination of production of embryogenic callus from meristematic explants is necessary. The meristematic explants taken from different locations were cultured on MS medium with 452.5 mol dichlorophenoxy acetic acid (2,4-D) and 14.7 mol 2 - IPA (2iP) and 3 g/l activated charcoal and early Callus producing embryogenic callus was evaluated . Due to the slow cells division of various tissues and meristematic regions, of different varieties of dates, within several times, re-culturing meristematic parts in different sizes from different meristem locations in four seasons, early callus producing embryogenic callus was evaluated. Table 2 shows the results of the effect of explants size and location and variety are at P=0.01 , but the impact of the explants taking time is insignificance at P=0.05. In addition, the interaction of variety and the explants place as well as the interaction of the explants location and size are significant, whereas, other mutual effects were not significant. Comparison of treatment mean scores indicated that the 8mm explants produced 347.96 mg callus and 4mm and 2mm explants were in the next rank (Table 5). These results accorded with Taha et al (2001) results. Among the explants location, a meaningful disparity was observed at the P=0.01 . The site C with mean core 448.519mg was the best (Table 4) . This agree with Al-Khayri et al (2004) result. Sites A and B were the next respectively (Fig.2). A meaningful disparity was identified between taking the explants at the P=0.05. The spring season with mean score 368.025mg callus per each explants had a better impact compared with other ties (Tale 3). This result agrees with Bonga and Aderkas (1988). After, summer, fall and winter were rated. About the impact of variety on callus production , a meaningful disparity was identified at the P=0.01 and the Khasouie variety with mean score 397.972 was better than other two varieties (Table 6).

Table 2. variance analysis of time, place, size and variety of taking explants and callus production and weight

Source of change	df	ss
		Callus production
Taking explants time	3	2193.70*
Explants place	2	901036.57*
Explants size	2	25237.78*
Variety	2	130175.42*
Explants time*explants place	6	755.03ns
Explants time* size	6	1027.47ns
Explants time*variety	6	609.43ns
Explants place*variety	4	2839.19**
Explants place * size	4	1485.98**
Explants size *variety	4	789.07ns
Explants time * place	12	920.35ns
time* place* variety	12	596.94ns
time *size*variety	8	976.39ns
time*size*variety	12	537.05ns
Time *place*size*variety	24	636.52ns
Change variance (CV)	6.609927	
Coefficient of determination	0.946828	

The impact of taking explants in callus production

As Table 2 shows, taking the explants time has a meaningful impact on the weight of produced callus at the P=0.05. Considering Table 3 in the present study, spring was the best time for taking the dates explants. The active times of the explants callus production may through some changes in the growth pattern like motivation of lateral branches or needle-shaped initials (10). In some tropical softwoods and hard woods, the stage the bust initiates its rapid growth in the spring is the best stage (11). Isolated branches of Sitka if collected in March, can easily produce adventitious shoots. The isolated stem segments of 20-year-old tree in Western Laryks during the growing season in comparison to the selected explants shows better reaction versus dormant buds during winter and fall (11). So, it seems more callus production in the growth season depends to more activities of mristematic tissues in the growing

season. This result is in accordance with Bonga and Aderkas (1991) results and contradicts with Chesick et al (1990).

Table 3. impact of taking time of the explants on wet weight of callus

Taking explants time	Mean wet weight of callus
spring	368.02a
summer	363.34ab
fall	361.72ab
winter	355.43b

Effect of the place of explants in producing callus

Staining and cut out method of the apical shoots allow providing an acceptable microscopic sample in a way that it is possible to isolate different part from the meristematic zone (figure 2). The results indicated that the zone C of the meristematic zone according to the table of mean score comparison (Table 3) due to having the highest rate of the meristematic tissue is the best place for callus production. Since the meristematic tissues have a high capacity of proliferation, they produce the heaviest amount of wet callus (16). The zone A consists of the meristemic tissues and leaf beginnings which caused the lowest wet weight of callus. However, Chukuemeka et al (2005) found out that when they used the apical meristem with some leaf beginning, they reached better results and explants which contained the apical meristem only were disappeared. The present research result is in accordance with the Fki et al (2003) results.

Table 4. the impact of the explants place on wet weight of callus

Explants place	Mean of wet callus weight
A	266.53c
B	371.34b
C	448.51a

The mean scores having identical letters in each column have no statistically meaningful variance in 1% level based on the Tukey-test.

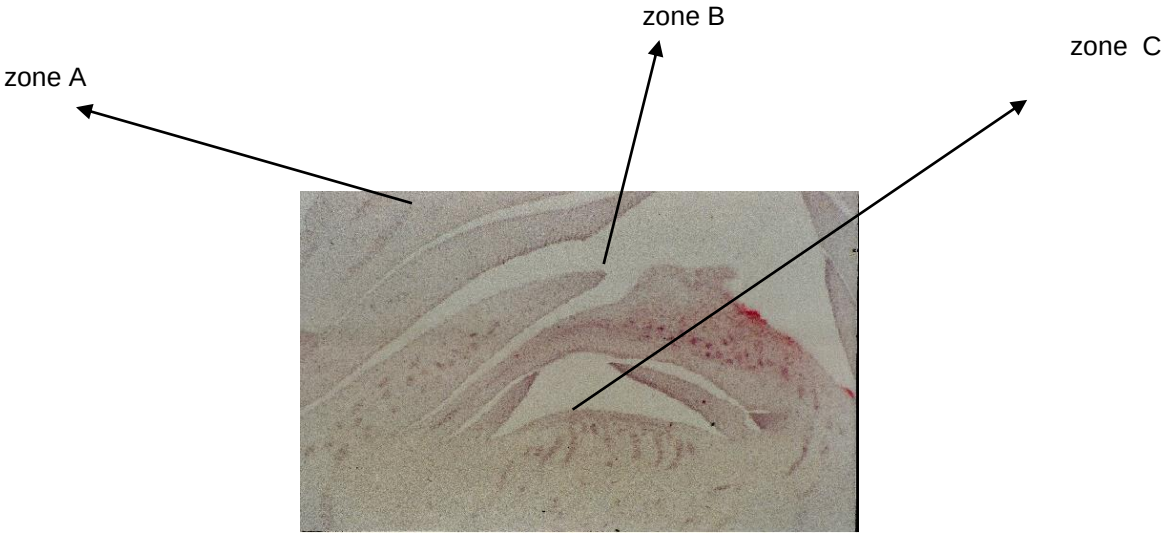

Figure 2. different zone of taking explants from apical shoots of dates

The impact of explants size on callus production

As Table 2 shows, the size of meristem explants of different varieties is effective on callus production. Table 4 shows that 8mm is the best size and this is because how much the explants is larger, it has more ability to regenerate (8,12). The smaller explants usually do not survive in the medium, but for obtaining explants free from any disease, explants with smaller size were used (8). Some of cells and tissues may be able to produce callus, but this is limited via the controlling features of the surrounding tissues(10). To remove such characteristics, miniature seized explants

were selected. To do so, the terminal lobs were used for culturing. These terminal lobs must preferably be cultured without initials; however these explants may less probably survive (15). In dates, explants sized 5-10mm indicated the best results (2,3,5,11). This result is in accordance with the Bonga and Aderkas (1991) results.

Table 5. impact of the explants size on callus wet weight

Explants size	Callus wet weight mean score
8	378.33a
4	360.10b
2	347.96c

The impact of variety on rate of callus tissue production

As the analysis of variance (Table 2) shows, the variety can affect callus tissue production in vitro from apical meristems of the dates. This impact was meaningful 1% level. In the present study as Table 4 shows, the Khasouei variety with mean score 97.972 mg gives the highest rate of callus production which indicates a significant variance in terms of rate of produced callus compare with other varieties (Khanizi with mean score 359.769 mg and Pyarom with mean score 328.657mg). The lowest callus production was for the Pyarom variety which after 12 weeks of culturing and 4 times re-culturing was obtained. This result is in accordance with Al-Khayri et al (2004), and Al-Khateeb (2006). The variance could be because of different response of tissues to the stress are imposed on them as a result of inadaptability of tissues in the medium depends on different varieties (9). That is, a variety experience stress in a specific medium and produce phenolic substance in a way that the other variety may show a good growth in the same medium without producing phenolic substance (22).the variety also leaves a meaningful impact on callus induction and asexual embryos and no comprehensive protocol has been provided so far dates proliferation through asexual embryogenesis. Compositions of the medium and time to plant differ from one variety to another (14). According to the varieties, different carbon sources improves asexual embryogenesis (28).

Table 6. the impact of variety on wet weight of callus

Variety	Mean of wet callus weight
Khasouei	397.97a
Khanizi	359.76b
Pyarom	328.65c

The mean scores having identical letters in each column have no statistically meaningful variance in 1% level based on the Tukey-test.

Mutual impact of the explants place and variety on callus production

Between different parts of a plant in response to in vitro culture, the differences in the physical conditions and the medium in particular, the type and amount of growth regulators such as auxin and cytokinin are important. It also depends on the level of IAA oxidase activity and mobility (3). According to the Table 2, in place A, the impact of variety one treatments is meaningful at the P=0.01. in all the varieties , place C is the best site for preparation of explants produce callus. As the explants place is closer to the meristem tissue, its regeneration activities will be greater. So, after place C, places A and B are in the next ranks respectively. As the results show, the Khasouie variety has the highest rate of callus production. These results accord with Al-Khayri and Al-Bahrany (2004) and Al-Khayri and Al-Bahrany(2001) results . But, contradict with Al-Hedramy and Baziz (1995) results.

Table 7. the mutual impact of the explants place and variety on wet weight of callus

variety	Location	callus weight (mg)
Khasouei	A	312.94a
	B	405.83a
	C	475.13a
Khanizi	A	257.63b
	B	368.47b
	C	453.19b
Pyarom	A	229.02c
	B	339.72c
	C	417.22c

The mean scores having identical letters in each column have no statistically meaningful variance in 1% level based on the Tukey-test.

Mutual impact of place and size of the explants in callus production

According to Table 8, in places A and B, the impact of explants taking treatments are meaningful at the P=0.01. in the place C which is the highest meristemic point , 8mm size showed the greatest rate of wet weight of callus compared with 2 and 4mm sizes. This disparity is meaningful the P=0.01. How much the biopsy place is more internal (C) and the size of explants is larger (8mm), more callus will be produced. This results accord with Chukuemeka et al (2005), Al-Khayry and Al-Bahrany(2001) and Al-Khatyb et al (2006) results. However, they contradict with Huong et al (1999) and Eshraghi et al (2005) results.

Generally, induction of growth in tiny explants like cell, cell mass and meristem is more complicated versus larger structures like leave, stem and tuber. The isolated explants can make more growth induction ad callus production due to food storage and enough hormons (10,16). In addition, larger explants with parenchymal tissue, vascular, and cambium have more ability in regeneration compared with smaller explants(10).

Tale 8. mutual impact of place and size of explants on callus wet weight

Size	callus weight (mg)	variety
[c] 245.69	2	
[b] 266.13	4	A
[a] 288.77	8	
[c] 357.08	2	
[b] 372.50	4	B
[a] 384.44	8	
[b] 441.11	2	
[b] 441.66	4	C
[a] 462.77	8	

Conclusion

Considering the results, for surface antiseptic, 40% bleach solution and for removing bacteria infection, increase of 100% Gentamycin and Kanamycin concentration per liter to the medium is suggested. For controlling the phenolic substances production, the antioxidants solution containing 100mg/l acid citric and 150mg/l ascorbic acid while cultivating and inside the medium besides 3mg/l activated charcoal give the best results. For production of callus tissue, the apical meristem, particularly the deeper section (C) with a size of 8 mm are the best explants. Spring was the appropriate time to create calluses from the apical meristem of dates. The greatest callus weight among the cultured varieties is for Khasouei with average 475.13mg (Fig.3). The medium of Murashige and Skoog (MS) had 452.5 µm dichlorophenoxyacetic acid (2,4-D), 14.7 µm (2-isopentenyl)adenine (2-iP) and 3g/l activated charcoal was the best medium for callus production.

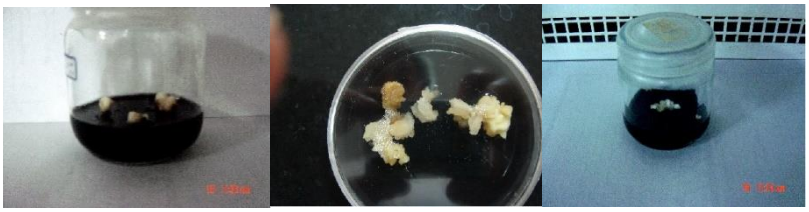

Figure 3. production of callus tissue from dried date palm, left to right: Pyarom , Khasouei , Khanizi

Acknowledgment

We wish to appreciate all authorities of the Agriculture Department of Hormozgan university for providing the researchers required facilities and the laboratory for conducting the present experiment.

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