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Culture Media Comparative Assessment of Common Fig (*Ficus carica* L.) and Carryover Effect

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Abstract

Production of common fig in Jordan has dropped more than 20-folds during the last five decades, due to several challenges, including biotic and abiotic stresses. The present study was conducted to asses a new protocol of shoot and callus development from fig apical buds. Explants of three local genotypes (Khdari, Mwazi and Zraki) were collected and grown in three culture establishment media: Murashige and Skoog (MS), Olive Medium (OM) and Woody Plant Medium (WPM). Two months later, shoot growth and callus development were measured, followed by transplanting into subculturing medium in test tubes with MS media. After additional two months, the same parameters were measured again. The results of the culture establishment showed that the highest shoot growth was obtained with OM and the highest callus development was obtained with WPM. On the other hand, the subculturing in MS medium showed prominent carryover effect of the first inoculation media, where the highest shoot growth was obtained in cultures transplanted from OM and the highest callus development was obtained in cultures transplanted from WPM. The present study delivers an improved protocol for establishment of common fig by tissue culture.

Keywords: Fig, in vitro, media, carryover effect.

1. Introduction

Common fig (*Ficus carica* L.) is one of the earliest cultivated plants. Fig fruits are known for their favorable taste and richness in nutrients and pharmacological compounds (Moon *et al*., 1997; Dhage *et al*., 2012). Common fig is native to the Mediterranean region, including Jordan (Sadder and Ateyyeh, 2006). However, the production of common fig in Jordan has dropped more than 20-folds during the last five decades. In addition, the production of common fig faces many challenges including biotic and a biotic stresses (drought, salinity, alkalinity, soil borne diseases and nematodes), (FAOSTAT, 2015). In Jordan, twelve local landraces are cultivated. Among which Khdari, Zraki and Mwazi are the most preferred and, together, they represent around 64% of the total cultivated fig in Jordan (Almugrabi and Anfoka, 2000). Increasing salinity has a negative effect on the number of shoot, shoot length and fresh and dry weight of common fig *in vetro* (Qrunfleh *et al.*). The number of fruits per shoot in the second crop of Khdari, Zraki and Mwazi are characterized by low, intermediate and high, respectively (Ateyyeh and Sadder, 2006b). The Zraki landrace produce the largest fig fruits in Jordan (up to 27.6 g) that are famous for their purple skin, while both Mwazi and Khdari have green fruit skin color (Ateyyeh and Sadder, 2006a).

Over the last few decades, tissue culture techniques have been used for rapid and large-scale propagation of a number of fruit trees (Bajaj, 1986; Zimmerman, 1986). The propagation by conventional method of (cuttings and grafting) is limited and slow. As those pieces can be obtained only from upright branches, which results in poor rooting and only 20–30% of the cuttings survive (Kumar *et al*., 1998).

In vitro culture of *Ficus* species has been widely studied as an alternative method for mass-scale production and high quality planting material (Rout *et al*., 2006). The successful results were obtained from using apical buds and shoots tip (Hepaksoy *et al*., 2006). The Murashige and Skoog (MS) (1962) culture medium is basically and widely used for plant tissue culture. Its components of salts are responsible for significant gains in tissue and cell development and growth. Additional media were also available for woody plants. The Woody Plant Medium (WPM) developed by Lloyd and McCown (1981) is the second most used medium for *in vitro* cultivation of woody species. It was developed for culturing shoots of woody plants and has found widespread use in the propagation of bushes and trees. WPM was shown to be beneficial for micropropagation of common fig (Brum, 2001; Mustafa *et al*., 2016). The olive medium (OM) is

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another medium developed for woody plants and utilized for micropropagation of olives (Rugini, 1984).

Most published articles of micropropagation of common fig focused on studying the effects of different concentrations of plant growth regulators on *in vitro* plant growth and development. However, the literature lacks comparative studies assessing multiple media. Therefore, we initiated this study to assess the effect of different media while ignoring the plant growth regulators' effects. Moreover, we are trying to move forward to build a strong tissue culture protocol for fig transplant production in Jordan.

2. Materials and methods

2.1. Explants Material

Healthy shoot cuttings with apical buds were collected from fig trees, which were planted in Shafa Badran Agricultural Station, the University of Jordan (32°3'36"N 35°55'22"E). Around 60-70 shoot cuttings (15 cm long) were utilized from each landrace of Mwazi, Zraki, and Khdari. Cuttings were transported in water for 1 hour. Apical buds were excised from the shoot cuttings and directly submerged in citric acid solution (1.5 mg/l). The explants were surface-sterilized with absolute ethanol for one min. Thereafter, the buds were surface-sterilized with commercial bleach (1.625% sodium hypochlorite) and few drops of tween-20 (Sigma, USA) for 15 min with continuous shaking. The samples were washed with sterile distilled water three times.

2.2. Culture Media

In the present study, three types of media were utilized, the first one was full strength MS medium (Murashige and Skoog, 1962), the second was WPM (Lloyd and McCown, 1981) and the third was OM (Rugini, 1984). In addition, all media contained 30 g/l sucrose and 1 mg/l 6-Benzylaminopurine (BAP) (Sigma, USA), the media were distributed in Petri dishes (20 ml each). The pH was adjusted in the range between (5.7-5.9) using 1 N NaOH and 1 N the HCl. The media were solidified with 1.2 g/l agar, and were sterilized by autoclaving at 121°C and 15 psi for 20 min.

2.3. Culture Establishment

The buds were inoculated in dishes. Surface sterilized buds were cultured on the surface of the three media working under aseptic conditions in laminar flow hood. Cultures were maintained in the growth chamber under a daily photoperiod of 16/8 (light/dark) provided by cool white fluorescent light and 23±2°C for two months.

2.4. Subculturing

After culture establishment, shoots and callus were transplanted to test tubes containing 10 ml MS medium for each tube in highly aseptic condition. The cultures were placed in the growth chamber under the same conditions used for culture establishment for additional two months.

2.5. Statistical Analysis

For culture establishment, explants were planted *in vitro* in 7 dishes (replications); each dish containing three buds in a Completely Randomized Design (CRD). Same design was maintained for subculturing. Data were analyzed using SPSS version 22 (2013) statistical analysis program. Means were separated by Tukey HSD with $p < 0.05$.

The shoot and callus development were evaluated at the end of both culture establishment and subculturing stages. Growth scales were used to assess callus development for size (1 = < 5 mm^2, 2 = 5-10 mm^2, 3 = 10-15 mm^2, 4 = 15-20 mm^2, 5 = 20-25 mm^2) and shoot growth for height (1= < 1 cm, 2 = 1-2 cm, 3 = 2-3 cm, 4 = 3-4 cm, 5 = 4-5 cm).

3. Results

3.1. Culture Establishment

Three different common fig landraces were used to obtain apical bud explants. Both callus development (Fig. 1.A) and shoot growth (Fig. 1.B) was achieved for the majority of cultures explants. Some cultures developed limited phenol oxidation in the medium surrounding the explant. However, it did not affect growth.

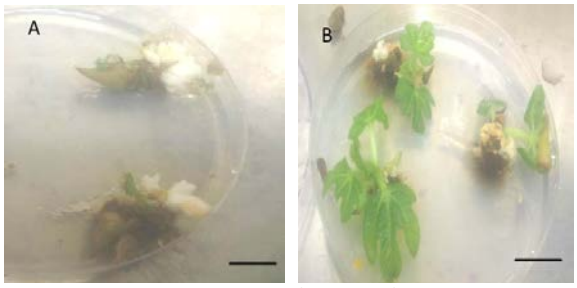

Figure 1. Culture establishment of common fig using bud explants 60 days old. A. Callus development in MS media, B. Shoot growth in WPM media, (Scale bar = 1 cm)

After two months of explant inoculation, when the data were combined, shoot growth in the landrace Khdari showed no significant differences between the three investigated media, whereas callus development was more prominent utilizing WPM when compared with the two other media (Table 1).For the Mwazi landrace, the highest significant shoot growth was achieved using OM compared to either MS or WPM. On the other hand, WPM gave the highest callus development compared to other media (Table 1). The shoot growth of the third landrace, Zraki, was not significantly different between the three investigated media, although the highest mean value was recorded for OM. The highest callus development in Zraki was achieved using WPM and the smallest was achieved using MS medium, while the OM resulted in an intermediate callus development which is not significantly different from those achieved by either WPM or MS medium (Table 1).

Table (1) Effect of different culture media on shooting and callus development of three different fig varieties namely; Khadri, Mwazi and zraki Means ± SD, Means followed by the different letters within the column are significantly different according to Tukey test at P ≤ 0.05

	Khdari		
Medium	**MS**	**OM**	**WPM**
Shoot growth	1.5^{a}±0.7	3.09^{a}±0.7	2.2^{a}±1.2
Callus development	1.5^{b}±0.7	1.54^{b}±0.7	3.0^{a}±0.65
	Mwazi		
Medium	**MS**	**OM**	**WPM**
Shoot growth	1.87^{b}±1.12	3.33^{a}±0.5	1.54^{b}±0.68
Callus development	1.5^{b}±0.75	1.0^{b}±0.0	2.81^{a}±0.75
	Zraki		
Medium	**MS**	**OM**	**WPM**
Shoot growth	2.0^{a}±1.0	3.5^{a}±0.7	2.0^{a}±0.86
Callus development	1.2^{b}±0.44	1.5^{ab}±0.7	2.22^{a}±0.44

When the data was combined for all three common fig landraces, the significantly highest shoot growth was achieved for OM (Fig. 2), which is more than 30% greater than the shoot growth achieved by either of the remaining media. In contrast, the combined callus development data for all three landraces, showed significantly highest figure for explants cultures over WPM with almost one-fold increase as compared to either calli grown over MS medium or OM (Fig. 2).

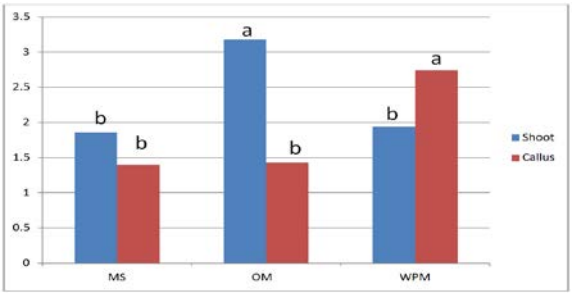

Figure 2. Effect of culture media on shoot and callus development of three different fig varieties of Jordan. Data represent means of all three landraces combined. Columns having different letters are significantly different

Furthermore, shoot growth showed no significant differences when comparing between the three landraces using combined data of all media (Fig. 3). On the other hand, the landrace Khdari revealed the most prominent callus development from bud explants, while the smallest development was recorded for Zraki common fig landrace (Fig. 3)

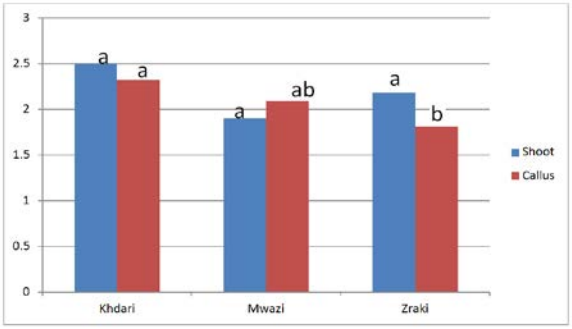

Figure 3. Effect of common fig landrace on shoot growth and callus development (Data represents means of all three culture media combined). Columns having similar letters are significantly not different

3.2. Subculturing

After culture establishment, the same explants were further transplanted into fresh MS medium to test the carryover effect of various culture media (Fig. 4). After a while the old shoots started to become discolored and died, however, new shoots grew from the bud base and those were considered for data measurement.

Figure 4. Shoot and Callus development of Mwazi buds after 60 days of .subculture. (Scale bar = 1 cm)

Two more months later, shoot growth in the landrace Khdari was highest in cultures transplanted from OM followed by WPM and then MS medium, with 3.5, 2.75 and 1.5, respectively (Table 2). Meanwhile, callus development was more progressive in cultures transplanted from WPM with at least 1.5-fold increase compared to the two other media. The shoot growth in the landrace Mwazi was highest in cultures transplanted from OM followed by MS medium and then WPM, with 4.12, 2.9 and 2.18, respectively (Table 2). On the other hand, there were no significant differences for callus development for cultures transplanted from any media. Likewise, Zraki shoot growth was highest in cultures transplanted from OM followed by MS medium and then WPM, with 3.6, 2.33 and 2.09, respectively (Table 2). The highest callus development in Zraki was achieved in cultures transplanted from WPM with more than 2-fold increase compared to cultures transplanted from either MS medium or OM.

Table (2) Media carryover effect on shoot growth and callus development of transplanted cultures. Means ± SD, Means followed by the different letters within the column are significantly different according to Tukey test at P ≤ 0.05

	Khdari		
Previous medium	**MS**	**OM**	**WPM**
Shoot growth	1.25^{b}±0.35	3.5^{a}±1.18	2.75^{ab}±0.65
Callus development	1.5^{b}±0.7	1.33^{b}±0.51	3.87^{a}±1.12
	Mwazi		
Previous medium	**MS**	**OM**	**WPM**
Shoot growth	2.9^{ab}±0.87	4.12^{a}±0.62	2.18^{b}±1.13
Callus development	2.2^{a}±1.03	1.5^{a}±0.57	2.12^{a}±083
	Zraki		
Previous medium	**MS**	**OM**	**WPM**
Shoot growth	2.33^{b}±0.75	3.60^{a}±0.54	2.09^{b}±0.7
Callus development	1.33^{b}±0.51	1.1^{b}±0.22	3.09^{a}±0.7

When the data were combined again for all three common fig landraces, the significantly highest shoot growth was achieved for cultures transplanted from OM (Fig. 5), which is this time around 40% greater than the shoot growth achieved from cultures transplanted from by either of the remaining media. In contrast, the combined callus development data for all three landraces showed significantly highest figure for explants cultures transplanted from WPM with almost one-fold increase as compared to either calli grown over MS medium or OM (Fig. 5).

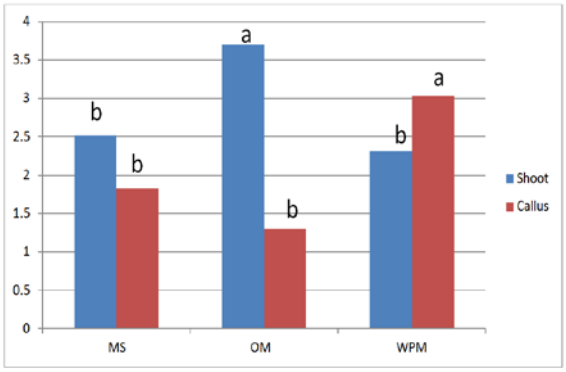

Figure 5. Effect of culture media before subculture on shoot growth and callus development (Data represents means of all three landraces combined). Columns having similar letters are significantly not different

Moreover, shoot growth showed no significant differences when comparing between the three landraces using combined data from cultures transplanted from all media (Fig. 6). Likewise, no significant differences were recorded between common fig landraces for callus development from bud explants transplanted from all cultures (Fig. 6).

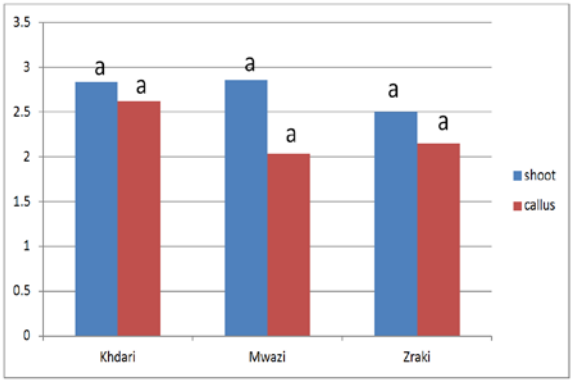

Figure 6. Effect of common fig landrace on shoot growth and callus development (Data represents means of cultures transplanted from all three media combined). Columns having similar letters are significantly not different

4. Discussion

When comparing the results of culture establishment data (Fig. 2) with culture subculture data (Fig. 5) after transplanting the cultures,the highest means of shoot growth was obtained in OM (3.18) and (3.7) for establishment and subculture, respectively ,and in the same time the highest means of callus development was clear by using WPM (2.74) and (3.03)for establishment and subculture, respectively. Similar trends can be found, emphasizing the carryover effect of initial culture medium. This is clear for shoot growth, which was outstanding in OM during culture establishment and after transplanting to new medium from OM. Similarly, callus development was outstanding in WPM during culture establishment and after transplanting to new medium from WPM.

MS, Knudson, WPM, and White and B5 media were assessed for micropropagation of common fig (Brum, 2001). Each medium was tested with four levels of sucrose (0, 15, 30 and 45 g/l). The results indicated that WPM supplemented with 20 g sucrose gave a high number of shoots and excellent growth of roots. Consequently, other reports have recommended WPM for common fig micropropagation (Fráguas *et al.*, 2004a; Fráguas *et al.*, 2004b; Mustafa *et al.*, 2016).

However, our results (Figs. 2 and 5) show that OM is a much better alternative to WPM. In olives, highest shoot regeneration was achieved from OM medium supplemented with thidiazuron or zeatin (Rugini, 1984; Mencuccini and Rugini, 1993; Zacchani and De Agazion, 2004). Unfortunately, only limited initiatives have assessed the potential of OM for woody plants other than olives. Shoot establishment and proliferation were achieved in guava cultured over OM (Papadatou *et al.*, 1990). In addition, OM was found to stimulate the development of new branches in *Juniperus phoenicea* (Loureiro *et al.*, 2007).

Our comparative investigation of MS medium, OM and WPM is basically a comparison of relative concentrations of macro- and micro-element as correlated to *in vitro* establishment of common fig. Important differences can be noticed when comparing the element concentrations in OM (Rugini, 1984), WPM (Lloyd and McCown, 1981) and MS medium (Murashige and Skoog, 1962). Boric acid concentration is almost twice in OM (12.2 mg/l) compared to either WPM or MS medium (6.2 mg/l). Boron (B), complexes with mannitol, mannan, polymannuronic acid, and other constituents of cell walls, is involved in cell elongation and nucleic acid metabolism (Taiz and Zeiger, 2002). Furthermore, magnesium sulfate concentration is four times higher in OM (732 mg/l) compared to both of MS and WPM (180.7 mg/l). Magnesium is required by many enzymes involved in phosphate transfer and is a major constituent of the chlorophyll molecule (Taiz and Zeiger, 2002). Moreover, potassium phosphate (Monobasic) concentration in OM (340 mg/l) is twice the level in either MS medium or WPM (170 mg/l). Phosphorus is component of sugar phosphates, nucleic acids, nucleotides, coenzymes, phospholipids and phytic acid. It has a key role in energy transfer reactions (Taiz and Zeiger, 2002). Another major difference is the higher concentration of zinc sulfate (Heptahydrated) in OM (14.3 mg/l) compare with the other two media (8.6 mg/l). Zn is a constituent of alcohol dehydrogenase, glutamic dehydrogenase, carbonic anhydrase (Taiz and Zeiger, 2002). Therefore, such elevated concentrations of these various elements in OM would be more beneficial in fulfilling shoot growth requirements in common fig as compared to WPM or MS medium.

Our data show that the WPM media gave the highest callus production in all common fig landraces investigated

in this study (Khdari, Zraki and Mwazi). This effect was further emphasized by the carryover effect on callus development of cultures transplanted from WPM compared to the other two media. This would be also promoted by the BAP supplied in our medium. BAP is one form of routinely applied cytokinins in plant micropropagation. They promote division, elongation and differentiation, delaying plant senescence, promote the breaking of the apical dominance and induce proliferation of axillary shoots (Taiz and Zeiger, 2002). Although BAP is the most widely used cytokinin, this does not mean it is ideal for all species. Similar to our results, callus development was found to be promoted in common fig using WPM supplemented with BAP (Fráguas *et al*., 2004b). The use of kinetin in the culture medium decreased the formation of callus in common fig (Jordan and Iturriaga, 1980). However, good callus induction was shown in the MS medium supplemented with 0.4 mg/l kinetin and 4.0 mg/l 2, 4-D (Danial *et al*., 2014). On the other hand, callus development is concentration dependent, fresh and dry callus weights were increased linearly with the increasing kinetin concentrations (Fráguas *et al*., 2004a).

Callus induction by WPM was also reported for other plant species, such as in *Barringtonia racemosa* (Behbahani *et al*., 2011) when compared with cultures grown over other media, like MS and B5. Furthermore, a fast callus growth was achieved by WPM medium with 2, 4-D. Farzinebrahimi *et al*. (2014) also found high percentage of callus formation with best dry and fresh weights to be formed on WPM supplemented with 2,4-D and NAA in *Gardenia jasminoides* Ellis. In fact, the concentration of various elements of three media investigated in our study, we can notice an elevated concentration of manganese sulfate in WPM (22.3 mg/l) as compared to both OM and MS medium (16.9 mg/l). In addition, Thiamine-HCl levels are 1.0, 0.1 and 0.5 mg/l in WPM, MS medium and OM, respectively. These important constituents with elevated levels in WPM may explain the promoting ability for callus development more than the other media. Thiamine was recorded to be essential for callus formation in other plant species including soybean (Ikeda *et al*., 1976) and date palm (Al-Kayri, 2001).

5. Conclusion

Our results revealed the importance of the medium per se and how much it could affect shoot growth and callus development. The data showed that the OM is more suitable for shoot growth of apical bud in common fig than either WPM or MS medium. On the other hand, WPM media were found to be crucial for callus induction and subculturing for different fig landraces. Moreover, it is important to consider the carryover effect of initial inoculation medium on both subsequent tissue growth and development.

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