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Screening Alfalfa Accessions for Regeneration Capacity

M.N. Barakat*, S. Milad and F.H. Khadr ****

**College of Agriculture and Veterinary Medicine, King Saud University, Qassim, Saudi Arabia*

***Tissue Culture Laboratory, Department of Crop Sciences, Faculty of Agriculture, University of Alexandria, Egypt*

Abstract. Nine cultivars of *Medicago sativa* and one accession of *M. arborea* were tested in six different media for their capacity to produce somatic embryos and plantlets from callus cultures. The effect of the interaction of genotype and culture medium on the initiation of callus from root, hypocotyl and cotyledon explants and the subsequent somatic embryogenesis was investigated. The results indicated that the number of embryos was significantly influenced by the variety and medium protocols and their interaction. The varieties responded differently to medium protocol with respect to number of plantlets.

Introduction

Medicago arborea has several agronomically valuable genes such as drought resistance and the ability to produce appreciable forage yield in the temperate climate which prevails in the Mediterranean area during the winter season when the cultivated *M. sativa* becomes dormant. Such traits of *M. arborea* would be of great value if they can be transferred to *M. sativa*. Unfortunately, sexual crosses between *M. sativa* and *M. arborea* fail to produce seeds due to some incompatibility barriers.

Somatic cell fusion facilitates the combination of plant genotypes that cannot be brought together by sexual crossing [1]. A prerequisite for the application of somatic hybridization to alfalfa improvement is the establishment of protocols which result in efficient somatic embryogenesis and subsequent plant regeneration in a wide range of commercially useful genotypes.

The induction of somatic embryogenesis from cell and tissue cultures has been reported to be dependent upon the genotype [2,3,4], medium protocol [5,6] and explant source [5,2].

This study was conducted to identify callus – inducible genotypes among Egyptian varieties to evaluate different media and to identify genotypes capable of somatic embryogenesis and subsequent plant regeneration.

Materials and Methods

Plant material

Seeds of eight *Medicago sativa* cultivars Siwa, Montakhab Nubaria, Pioneer 572m Amador, GT 13RT, El-Wadi El-Gedeed, Wahat, Montakhab Esmaelia and the accession of *M. arborea* were obtained from the Forag Crop Section, Field Crop Institute, Agricultural Research Center, Giza, Egypt. Seeds of *M. Sativa* cv. European Lucerne were obtained from the Plant Genetic Manipulation Group, Botany Department, Nottingham University, England. Seeds were surface sterilized in 99% ethanol for 30 seconds followed by 15 min 0.1% $HgCl_2$ and therafter washed with six changes of sterile tap water. Seeds were germinated on agar solidified (0.8% W/V) based medium (MS) [7] containing 3% sucrose, but lacking growth regulators and incubated in the light (2000 Lux, daylight fluorescent tubes) at 23 ± 2°C.

Callus induction

Three different explants were prepared from 7 days seedlings. (1) The cotyledons were cut into half longitudinally. (2) Hypocotyl segments of 5-10 mm long were excised. (3) The roots were cut into segments of 5-10 mm length. The three explants of the same seedling were placed, on to the surface of a solidified culture medium, and, in one petri dish. Twenty seedlings from each variety were tested for each medium protocol. The six media protocols used in this study are listed in Table 1. The dishes were incubated under 16 h illumination (2,000 lux, day light fluorescent tubes) at 25 ± 2°C.

Four weeks after incubation, the fresh weight of the initiated callus derived from different explant types for the six media per variety was recorded under sterile conditions as average of twenty seedlings.

Somatic embryogenesis and plant regeneration

Calli derived from different explant types were transferred to the six media sequences (Table 1) for the induction of somatic embryogenesis. At the end of culture sequences, the somatic embryos and subsequent plant recovery were counted as average of twenty seedling. The plantlets were then transferred to a mist chamber, in the greenhouse, for two weeks and then grown as for plants derived from seeds.

Table 1. Medium protocols used to determine callus induction response, embryogenic response and shoot regeneration in some *Medicago sativa* varieties and *M. arborea*

Protocol	Medium sequence (day of culture) [growth regulator concentration mg. L^{-1}]			
	UM (28)	UM (28)	MS (28)	MS (14-28)
A	2.0 2.4-D	2,0 2,4-D	0.05 NAA	[0]
	0.25 Kinetin	0.25 Kinetin	0.5 6-BAP	
B	MS (28)	MS (28)	SH (28)	B 5 (14-28)
	2.0 2,4-D	2.0 2,4-D	1.6 g $(N.H_4)_2SO_4$	[0]
	0.2 Kinetin	0.25 Kinetin	5.75 g proline	
C	MS (28)	MS (28)	SH (28)	B5 (14-28)
	2.0 2,4-D	[0]	1.6 g $(NH_4)_2SO_4$	[0]
	0.2 Kinetin	0.2 Kinetin	5.75 g proline	
D	SH (28)	SH (28)	Boi2y (28)	B 5 (14-28)
	4.7 NAA	11.1 2,4-D	[0]	[0]
	2.2 Kinetin	1.1 Kinetin		
E	SH_{II} (28)		Boi2y (28)	Boi2y (7-14)
	2.0 2,4-D		[0]	[0]
	2.0 Kinetin			
F	Bo (28)		Boi2Y (28)	Boi2y (7-14)
	2.0 2,4-D		[0]	[0]
	2.0 kinetin			
	2.0 NAA			

Media abbreviations: UM = Uchimiya and Murashige [16],
MS = Murashige and Skoog [7], SH = Schenk and Hildebrandt [17],
B_5 = Gamborge *et al.* [18], Boi2y = Saunders and Bingham [19],
Bo = Bingham *et al* [2].

Statistical analysis

Data were statistically analyzed according to Snedecor and Cochran [8]. Total embryos and total plantlets were analyzed following a square root + 0.5 transformation. Comparisons among means were made via the least Significant Difference multiple range test.

Results and Discussion

Callus induction

The analysis of variance for callus weight presented in Table 2 indicated that callus weight was highly significantly influenced by varieties, medium protocols and explants. All the two way interactions and the three way interactions were highly significant.

The Um medium containing 2.0 mg/L 2,4-D and 0.25 mg/L kinetin gave the optimum callus induction in most varieties of *Medicago* species (Table 3). On the other hand callus growth was minimal on Bo medium containing 2.0 mg/L 2,4-D, 2.0 mg/L kinetin and 2.0 mg/L NAA.

The *Medicago arborea* produced the highest significant callus weight (0.626 g) across media (Table 3) which was also higher than that produced from any variety of *M. sativa*. Among *M. Sativa* varieties Montakhab Nubaria gave significantly the highest callus weight (0.547 g). Wahat has significantly the lowest callus (0.222 g) and it was insignificantly different from El-Wadi El-Gedeed (0.251 g) and GT 13 RT (0.243 g).

The response for callus induction varied according to medium composition. Table 3 indicates that UM medium gave the highest average weight of callus (0.500 g) accross varieties and it was significantly superior to all other media. On the other hand, Bo medium was significantly inferior to all other tested media. The data (Table 3) also revealed that there was an interaction between the variety and medium. *M. arborea* gave the highest callus weight when cultured on UM or SH medium (0.879 and 0.839 g, respectively). Nevertheless, there were no significant differences among those two treatments and the average of Montakhab Nubaria with SH II medium and Siwa with UM medium (0.833 and 0.802 g, respectively). These four treatments significantly produced the highest callus weight. While Bo medium gave the lowest weight with most of the varieties.

The cotyledon explants significantly gave the highest response to callus induction (0.481 g) as compared to the other two sources (Table 3). However, the difference between hypocotyl and root in callus induction was not significant. There was a highly significant interaction between the variety and explant. The hypocotyl of *M. arborea* gave the highest weight of callus (0.945 g). For the varieties of *M. sativa*, the highest weight of callus was obtained from the cotyledon explant, except for Amador and Wahat where differences between cotyledon and root explants were insignific-

Table 2. Analysis of variance for callus weight (grams), number of embryos and number of regenerated plants

Source of variance	D.F.	M.S. of callus weight	D.F.	M.S. of no. embryos[a]	M.S. of no. plantlets[a]
Treatments	149	1.61 **	179	0.168	0.088 **
Varieties (A)	9	5.76 **	9	0.34 **	0.165 *
Protocols (B)	4	9.40 **	5	0.68	0.279 **
Explants (C)	2	9.34 **	2	0.31	0.082
A x B	36	1.03 **	45	0.29 **	0.175 **
A x C	18	1.95 **	18	0.10	0.044
B x C	8	3.91 **	10	0.08	0.040
A x B x C	72	0.39 **	90	0.08	0.040
Error	2850	0.063	3420	0.126	0.079
Total	2999		3599		

* Significant at 0.05 level

** Significant at 0.01 level

a Data were transformed to $\sqrt{x + 1/2}$

Table 3. Mean values for callus weight (gram) as influenced by varieties, medium protocol and explants and their interactions

Species	Variety	Medium protocol						Explant		
		UM	MSK	SH	SH_{II}	B_o	Variety mean	Cotyledon	Hypocotyl	Root
M. sativa	Siwa	0.802	0.454	0.218	0.566	0.098	0.428	0.688	0.308	0.287
	Montakhab Nubaria	0.662	0.412	0.510	0.833	0.316	0.547	0.785	0.515	0.339
	Pioneer 572	0.584	0.524	0.260	0.445	0.149	0.392	0.570	0.297	0.310
	Amador	0.532	0.438	0.079	0.207	0.224	0.296	0.393	0.163	0.332
	GT 13 Rt	0.279	0.330	0.219	0.262	0.125	0.243	0.349	0.146	0.234
	El-Wadi-El-Gadeed	0.303	0.347	0.232	0.253	0.117	0.251	0.376	0.182	0.193
	European Lucerne	0.525	0.545	0.315	0.638	0.112	0.427	0.568	0.303	0.409
	Wahat	0.208	0.275	0.237	0.298	0.095	0.222	0.217	0.180	0.270
	Montakhab Esmaelia	0.230	0.423	0.216	0.276	0.177	0.264	0.387	0.186	0.220
M. arborea		0.879	0.446	0.839	0.570	0.398	0.626	0.473	0.945	0.461
	Mean	0.500	0.419	0.312	0.435	0.181	–	0.481	0.323	0.306
Explant										
	Cotyledon	0.806	0.669	0.289	0.467	0.178				
	Hypocotyl	0.424	0.277	0.305	0.421	0.185				
	Root	0.270	0.311	0.344	0.417	0.185				

$L.S.D._{(0.05)}$ for variety means = 0.040
$L.S.D._{(0.05)}$ for medium means = 0.028
$L.S.D._{(0.05)}$ for explant means = 0.022
$L.S.D._{(0.05)}$ for variety x medium interaction = 0.090
$L.S.D._{(0.05)}$ for variety x explant interaction = 0.070
$L.S.D._{(0.05)}$ for medium x explant interaction = 0.490

ant. Differences in callus weight between hypocotyl and root explants were related to their varieties.

The first order interaction between medium protocol and explant was highly significant. Cotyledon explant gave the highest weight of callus (0.806 g) with UM medium, whereas all the three explants gave the lowest weight with Bo medium (Table 3).

The obtained results provide an indication of the relative importance of genotype and media effect on culture response. These results showed that the growth rate of the callus was dependent on the varieties, the explant source and the culture medium employed. Such results are in agreement with Kao Michayluk [9] who reported that genotypic and varietal differences are important factors in obtaining successful results in tissue culture of forage plants.

Embryogenesis and Plant Regeneration

Statistical analysis of somatic embryogenesis responses revealed highly significant difference among varieties and among medium protocols (Table 2). The varieties x medium protocols interaction was significant at the 0.10 probability level for number of embryos. However, there were no significant differences among root, hypocotyl and cotyledon explants with respect to the number of embryos.

Differences in response to ability to form somatic embryos were observed among different varieties in the present investigation (Table 4 and 5). The best response was obtained with the varieties European Lucerne, Montakhab Numbaria and Pioneer 572 as well as the wild species. The callus tissue on UM medium (protocol A) gave rise to clusters of green embryoids. Other embryoids were also observed scattered on the callus surface. The 3-step induction method (protocol A and B) which use a high auxin (2,4-D) to a low cytockinin (kinetin) ratio of growth regulators were the most effective for embryo induction.

When the embryos were placed onto hormone free medium (Table 1) embryoids germinated into plantlets. However, some embryos did not develop, some grew into callus, some proliferated into more embryos and some grew only into roots. It was also observed that germination of embryos and success of plant recovery varied among varieties. The efficiency of embryo germination and subsequent plant recovery ranged from 0.0% to 100% (Table 6).

Table 4. Mean values for number of embryos and number of plantlets as influenced by varieties and medium protocols and their interactions

Species	Variety	Medium protocol													
		A		B		C		D		E		F		Variety mean	
		E*	P*	E	P	E	P	E	P	E	P	E	P	E	P
M. sativa	Siwa	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.710
	Montakhab Nubaria	0.924	0.811	0.930	0.899	0.733	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.786	758
	Pioneer 572	0.803	0.742	0.725	0.710	0.729	0.710	0.710	0.710	0.800	0.776	0.750	0.710	0.753	0.726
	Amador	0.710	0.710	0.752	0.770	0.710	0.710	0.710	0.710	0.710	0.710	0.729	0.710	0.720	0.715
	GT 13 Rt	0.757	0.729	0.729	0.719	0.710	0.710	0.710	0.710	0.719	0.710	0.725	0.710	0.725	0.715
	El-Wadi-El-Gedeed	0.710	0.710	0.719	0.710	0.734	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.715	0.710
	European Lucerne	0.178	1.075	0.744	0.710	0.719	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.795	0.771
	Wahat	0.744	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.710	0.741	0.710	0.721	0.710
	Montakhab Esmaelia	0.710	0.710	0.729	0.719	0.798	0.752	0.710	0.710	0.710	0.710	0.710	0.710	0.728	0.718
M. arborea		0.753	0.719	0.885	0.796	0.771	0.710	0.719	0.710	0.710	0.710	0.746	0.710	0.764	0.726
Protocol	Mean	0.800	0.763	0.763	0.742	0.732	0.714	0.711	0.710	0.720	0.717	0.724	0.710	–	–

* E = number of embryos, P = number of plantlets

$L.S.D._{(0.05)}$ for variety means = 0.052 and 0.032 of number of embryos and plantlets, respectively.

$L.S.D._{(0.05)}$ for medium means = 0.040 and 0.041 of number of embryos and plantlets, respectively.

$L.S.D._{(0.05)}$ for variety x medium interaction = 0.127 and 0.100 of number of embryos and plantlets, respectively.

Table 5. Number of embryos obtained by using different explants and different medium protocols for *Medicago sativa* varieties and *M. arborea*

Variety	Medium* protocol	Explant			Total
		Cotyledon	Hypocotyl	Root	
Montakhab	A	26 (4)	27 (4)	0	53
Nubaria	B	15 (1)	74 (2)	0	89
	C	1 (1)	2 (2)	0	3
Pioneer 572	A	8 (3)	0	5 (4)	13
	B	0	0	2 (1)	2
	C	0	3 (1)	0	3
	E	0	37 (1)	0	37
	F	9 (1)	0	0	9
Amador	B	0	10 (1)	0	10
	F	3 (1)	0	0	3
GT 13 Rt	A	4 (2)	0	3(1)	7
	B	3 (1)	0	0	3
	E	1 (1)	0	0	1
	F	2 (1)	0	0	2
El-Wadi-El-Gedeed	B	0	1 (1)	0	1
	C	4 (1)	0	0	4
Europeane	A	35 (1)	76 (3)	184 (2)	295
Lucerne	B	5 (2)	0	0	5
	C	1 (1)	0	0	1
Wahat	A	5 (2)	0	0	5
	F	0	6 (1)	0	6
Montakhab	B	3 (1)	0	0	3
Esmaelia	C	4 (2)	18 (1)	0	22
M. arborea	A	3 (2)	3 (3)	1 (1)	7
	B	6 (4)	23 (6)	9 (2)	38
	C	0	9 (4)	0	9
	D	0	0	1 (1)	1
	F	5 (1)	1 (1)	0	6

Figures between brackets represent original number of seedlings which gave embryos

* See Table 1 for medium protocol

Table 6. Percentage of embryos which developed into plantlets

Species	Variety	Medium* protocol	Explant		
			Cotyledon	Hypocotyl	Root
M. sativa	Montakhab	A	50.0	51.9	+
	Nubaria	B	73.3	83.8	–
	Pioneer 572	A	37.5	–	20.0
		E	–	56.8	–
	Amador	B	–	50.0	–
	GT 13 Rt	A	0	–	100.0
		B	33.0	–	–
	Europeane Lucerne	A	60.0	64.5	76.6
	Montakhab	B	33.3	–	–
	Esmaelia	C	0	55.5	–
M. arborea		A	33.3	0	0
		B	0	39.1	33.3

* See table 1 for medium protocols

\+ No embryo was obtained

The number of plantlets was significantly influenced by varieties and highly significantly affected by medium protocol (Table 2). The variety x medium interaction was highly significant.

It was evident from Tables 4 and 6 that European lucerne and Montakhab Nubaria recorded the highest significant number of plantlets. In contrast, Siwa, El-Wadi El-Gedeed and Wahat did not give any plantlets. Also, data indicated that medium protocols A and B produced the highest number of plantlets. However, differences between medium protocol B and the other medium protocols were not significant (Table 4).

Morphological variations have been observed after transferring embryos in some medium protocol. For example, some of the embryos obtained from *M. sativa* varieties montakhab nubaria with medium protocol B, montakhab esmaelia with medium protocol C and *M. arborea with medium protocol B were multiplied or pro-*

liferated when transferred onto SHP medium. This observation was similar to those reported by Santos et al. [10] and Lupotto [11].

Results of this investigation show that there are strong cultivar X medium interactions for both somatic embryogenesis and plant regeneration. Variety differences have been reported as important variables in the regneration of several species [12,13,14]. Genotypic variation in embryogenesis is a widespread phenomenon in alfalfa [3,5,15].

The present investigations are however, of a preliminary nature and were designed to establish the culture conditions conductive for callus initiation and subsequent plant regeneration. The results of embryogenesis with *M. sativa* and *M. arborea* explants and callus provided useful information for subsequent investigations regarding plant regeneration from cultured protoplasts.

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حصر أصناف البرسيم الحجازي للقدرة على إنتاج نباتات من زراعة الأنسجة

محمد نجيب بركات*، سناء إبراهيم ميلاد محمد وفؤاد حسن خضر****

** كلية الزراعة والطب البيطري، جامعة الملك سعود، القصيم، المملكة العربية السعودية*

*** ومعمل زراعة الأنسجة، قسم المحاصيل، كلية الزراعة، جامعة الأسكندرية، جمهورية مصر العربية*

ملخص البحث. تم تقويم ثمانية أصناف من البرسيم الحجازي *M. sativa* وسلالة واحدة من *M. arborea* من حيث القدرة على إنتاج الكالس وتكوين الأجنة وإنتاج النباتات الكاملة، وذلك باستخدام ثلاثة أجزاء نباتية مختلفة من البادرة (الجذر ـ السويقة الجنينية السفلي ـ الفلقات)، وستة تتابعات من البيئات. وقد أظهرت النتائج مايلي:

١ ـ تأثرت صفة وزن الكالس وعدد الأجنة وأعداد النباتات الناتجة من إنبات الأجنة معنويًا بالأصناف، وتتابعات البيئات والتفاعل بينهما.

٢ ـ أعطى النوع *M. arborea* أعلى وزن كالس ومن بين أصناف *M. sativa* أعطى منتخب نوبارية أعلى وزن كالس.

٣ ـ أصناف البرسيم الحجازي *M. sativa* الأوربي ومنتخب نوبارية وبيونير ٥٧٢ أعطت أحسن استجابة في عدد الأجنة.

٤ ـ أعطت الأصناف الأوربي ومنتخب نوبارية التابعة للنوع *M. sativa* أعلى عدد من النباتات.

٥ ـ أمكن الحصول على العديد من النباتات من الأصناف المختلفة ومعظمها وصل إلى طور الإزهار.