DEVELOPMENT OF AN EFFICIENT REGENERATION SYSTEM FOR EGYPTIAN CULTIVAR(S) OF BARLEY (*Hordeum vulgare* L.)

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Cultivated barley (*Hordeum vulgare* L.) belongs to the Triticeae in the grass family, Poaceae. Barley ranks as the world's fourth most important cereal crop after wheat, maize and rice. It is grown over a wide environmental range than any other cereal crop. It is more tolerant to drought, summer frosts, saline and alkaline soils than other cereals; the grain is used for human food, animal feed and malt (FAO, 1998). Although barley is an important member of the cereal crops, it lags behind in gene transfer technology with regard to the introduction of elite agronomic traits such as disease and herbicide resistance. Non-sexual transfer of DNA to cereal species such as *Hordeum vulgare* L. has historically been problematic, chiefly because of problems encountered during attempts to regenerate cultured transgenic cells. Frequently, few or no plants can be recovered following selection for transformed cells, or only albino can be recovered (Lemaux *et al*., 1999). The recovery of fertile plants from transgenic callus is a critical component of many cereal transformation systems (Bregitzer *et al*., 1998; Hussein *et al*., 2004; Assem *et al*., 2008). Efficient regeneration of plants from transformed embryogenic callus is often limited to specific genotypes that exhibit vigorous plant regeneration (Bregitzer *et al.*, 1998).

Improved plant regeneration from elite barley cultivars will facilitate their genetic transformation. The media composition is an important key factor influencing the regeneration ability of different genotypes. Different media compositions have been applied by Bregitzer (1992) and Hussein *et al.* (2004). Therefore, it is necessary to optimize the regeneration conditions for the Egyptian cultivars.

The objectives of the present investigation were: to establish an efficient regeneration system for some Egyptian barley genotypes using immature embryos as explants, and to evaluate the effect of different media composition on the embryogenic

response and regeneration ability of the barley genotypes.

MATERIAL AND METHODS

Plant materials

Four Egyptian barley (*Hordeum vulgare* L.) genotypes including two commercially important cultivars (Giza 123 and Giza 125) and two promising breeding lines (line 9 and line 11) were used in this investigation. Barley seeds were kindly provided by the Barley Department, Field Crops Research Institute, ARC, Giza, Egypt. Seeds of the four barley genotypes were sown in the field at 17 days intervals to provide continuous source of immature embryos. Spikes with immature embryos, about 1.5 to 4 mm in length, were harvested from field-grown plants ~14 days post anthesis. Caryopses were surface sterilized by soaking for 1 min in 70% ethanol, 5 min in 8% sodium hypochlorite containing few drops of Tween 20, followed by 5 min rinse with distilled water. The last step was repeated 5 times.

Media composition and preparation

Four different MS-based media were utilized. BS medium is the basal salt medium described by Murashige and Skoog (1962), M1 medium is the original medium described by Bregitzer (1992). In addition, two other media, i.e., LD improved medium described by Dahleen & Bregitzer (2002) and a modified EB medium were used in an attempt to improve the efficiency of regeneration. The media compositions are detailed in Table (1). These media differed in the concentration of micronutrients ($CuSO_4$, H_3BO_4, and $FeSO_4$) and/or growth regulators and the type of carbon source. LD media and EB medium contained an increased concentration of $CuSO_4$ and H_3BO_4 (5.0 µM vs 0.1µM and 0.75 mM *vs* 0.1 mM, respectively) and a reduced concentration of Fe_2SO_4 (0.05 mM *vs* 0.1 mM) compared to the other two media. Moreover, BS, M1, and LD media were supplemented with 3 mg/l 2, 4-D (2,4 dichlorophenoxyacetic acid), while EB medium contained 2mg/l Dicamba (3,6 dichloromethoxybenzoic acid) in the callus induction and maintenance stages. In addition, sucrose was substituted by maltose in LD medium and EB medium. The method used to prepare the media was modified according to Bregitzer *et al*. (1998) method, by autoclaving the following components separately: (1) Iron and potassium phosphate, (2) Maltose or sucrose, (3) all other ingredients. The medium was solidified with 3.5 g/l phytagel. The three parts were cooled to 65°C, mixed and then poured in the culture plates.

Callus initiation and maintenance

Callus was initiated from longitudinally bisected immature zygotic embryos along the root-shoot axis. Thirty explants (bisected immature embryo) were cultured in each plate (100 x 25 mm) containing ~ 50 ml medium and kept in the dark at 22°C. The total number of tested explants from each genotype was 600 (300 per experiment). After two weeks on

callus induction medium the embryogenic friable calli were broken into small pieces and subcultured onto freshly prepared callus induction medium, the last step was repeated one more time. After six weeks on callus induction media, the growing calli were transferred to the corresponding maintenance medium and placed under dim light with 2 x 10^3 Lux with 16/8 hr (light/dark) photoperiod at 22°C in an incubator. As shown in Table (1), all the maintenance media have the same composition as their respective callus induction media and supplemented with 0.1 mg/l BAP (6-benzylaminopurine) except BS medium.

Plant regeneration

After approximately four weeks on maintenance medium, the growing calli were transferred to the regeneration media and placed under bright light with 6x10^3 Lux 16/8 hr (light/dark) photoperiod at 22°C. Subculture onto a fresh medium was performed every two weeks. Shooted calli were then transferred to the rooting medium composed of half strength MS medium, containing sucrose as a carbon source and devoid of hormones. On this medium, the shoots were allowed to develop into plantlets in jars at 22°C with 16/8 hr photoperiod. The regenerated plantlets were transferred to pots in the Conviron® after washing the roots with an anti-fungal agent. The pots contained 1:1:1 mixture of sterilized sand, peatmoss and soil. The Conviron® was adjusted at 22°C with 6x10^3 Lux 16/8 hr (light/dark) photoperiod and 80% relative humidity (RH). Pots were irrigated with half strength of Hoagland solution.

Statistical analysis

Data were collected from the regeneration experiments and analyzed with M state C program.

RESULTS AND DISCUSSION

Callus initiation and maintenance

Four different callus induction media were employed to investigate the efficiency of callus formation from bisected immature embryos of the four barley genotypes. Two replicates with a total of 600 explants for each genotype were cultured on each of the callus induction media. The scutellum was in contact with surface of the medium. On the average 30 explants were cultured per plate. Within few days after culturing, callus was initiated from the bisected immature embryos of the four barley genotypes. Two morphotypes of callus were observed, i.e., embryogenic and non-embryogenic calli. The non-embryogenic callus was compact and slow growing. While, embryogenic callus ranged from white color, fine divided and relatively friable as revealed by Giza 123 and Giza 125 on LD medium, to callus that was compact, nodular, yellowish white as observed on EB medium with Giza 123 and Giza 125. As shown in Tables (2 and 3), significant increase in the number of embryogenic calli was observed for the four genotypes when using LD and EB media. However, the mean number of embryogenic calli developed

by the two genotypes Giza 123 and Giza 125 on LD medium was higher than on EB medium (251.0 vs. 248.0 and 215.0 vs. 162.5 for Giza 123 and Giza 125, respectively). While, the other two genotypes (Line 9 and Line 11) produced a higher average of embryogenic calli on EB medium than on LD medium (210.0 vs. 200 and 110.5 vs. 48 for Line 9 and Line 11, respectively). Nevertheless, the difference between the average number of embryogenic calli on these two EB and LD media was not significant for the three genotypes Giza 123, and Giza 125 and Line 9.

Data presented in Table (2) also revealed that the genotype Giza 123 developed the highest mean number of embryogenic calli on the four media (173.5) followed by the two genotypes Giza 125 (128.3) and Line 9 (125.3). While, Line 11 produced the lowest mean number of embryogenic calli (49.88). This clearly demonstrated that the formation of embryogenic calli is a genotype dependent character. These results are in good accordance with those obtained for most cereal species, confirming that regeneration is strongly genotype-dependent (Maddock, 1985; Fennell *et al*., 1996; Bregitzer *et al*., 1998; El-Itriby *et al*., 2003). Most of the calli formed by genotypes Giza 123, Giza 125 and Line 9 on LD and EB media were friable, yellowish-white and embryogenic. This type of callus was able to reveal all the embryogenic developmental stages (Fig. 1).

Data presented in Table (3) indicated that EB medium gave the highest mean frequency of embryogenic calli (182.8) across the four genotypes. This was followed by LD medium (178.8) with no significant difference. While, this value was significantly reduced on M1 medium (99.88) and the lowest value was exhibited by BS medium (15.75). These results pointed out that modifying the media composition, mainly by decreasing the concentration of ferric sulfate and increasing the concentration of copper sulfate and boric acid, greatly improved the average number of embryogenic calli in the four barley genotypes. Moreover, the substitution of the growth regulator, i.e. 2, 4-D by Dicamba improved significantly the mean number of embryogenic calli in the recalcitrant Line 11 genotype. These results are in agreement with those of Tiidema and Truve (2004). They pointed out that the regeneration medium containing Dicamba produced significantly higher rates of callus induction, callus growth and regeneration percentage of barley plants however, but in contrast to our results, it yielded also many albino plants. Moreover, Assem *et al.* (2008) stated that Dicamba compound promoted fast differentiation of four Egyptian maize inbred lines and led to increase the number of shoots in comparison to 2,4-D.

After six weeks on callus induction medium, all the embryogenic calli were transferred to the corresponding maintenance media (Table 2). The maintenance medium for LD, EB and M1 media was identical to the callus induction stage but supplemented with 0.1 mg/l of the benzylaminopurine (BAP) to promote shoot

initiation. While, in the case of the BS medium, the concentration of the 2,4-D synthetic auxin was reduced into half in the maintenance medium.

As shown in Table (2), not all the induced calli were able to survive on maintenance media. The results also revealed that the addition of 0.1mg/l BAP to the maintenance media led to an increase in green shoot initiation. This finding is in good agreement with the results of Cho *et al*. (1998) and Dahleen and Bregitzer (2002). They concluded that the inclusion of BAP in the maintenance medium increased the frequency of highly regenerative green structures and green plant regeneration compared with cultures on medium lacking BAP.

Plant regeneration

After four weeks on maintenance medium, the calli with green shoots were transferred to regeneration media for shoot elongation. Then the regenerated shoots were transferred to the rooting media. Non-regenerated plantlets were discarded during subculture. Results presented in Table (2) revealed that the highest regeneration frequency has been obtained by Giza 123 on EB medium (27.20%) followed by LD medium (13.32), while medium BS expressed the lowest percentage in Giza 123 (1.00%). Similarly, the cultivar Giza125 revealed the highest regeneration frequency on EB medium (18.60%) followed by LD medium (12.00%), while, BS medium failed to regenerate plantlets. Breeding Lines 9 and 11 regenerated plants only on LD and EB media at low percentages (9.32 and 1.32 for Line 9 and 9.32 and 6.00 for Line 11). While, these two Lines did not produce any plants on M1 medium and BS medium. The mean values of regeneration frequency over all the four media used for Giza 123 and Giza 125 (11.03% and 8.98%., respectively) was significantly higher than those for breeding Lines 9 and 11 (2.66% and 3.83%, respectively).

Statistical analysis of variance presented in Table (4) revealed that the mean square numbers of embryogenic calli and regenerated plantlets among genotypes and media were significant. Moreover, the interaction between genotype and media revealed significant effect on embryogenic calli and regenerated plantlets. It revealed that shooting and rooting quality of Giza 123 and Giza 125 were better on EB medium than on LD medium. Stages of barley regeneration system of genotype Giza 123 on EB medium are illustrated in Fig. (2). Therefore, it could be concluded that the genotype of the cultivar and the nutrient composition of growth medium exert major influence on the *in vitro* performance of barley. These results are in consistence with those of Hanzel *et al*. (1985); they noted significant genotype by medium interactions for callus initiation among 96 genotypes. Similarly, Luthers and Lörz (1987) found that only 1 of 36 genotypes formed embryogenic callus on B5 medium. Moreover, Bregitzer (1992) and Dahleen and Bregitzer (2002) and Hussein *et al*. (2004) found that the frequency of embryogenic callus induction, the morphology, friability of embryogenic

calli, the relative growth rate of embryogenic calli and regenerability were influenced by genotype, culture medium and genotype x culture medium interaction.

The present study suggested that the development and use of genotype-specific media regime and regeneration protocols can enhance plant regeneration of Egyptian barley genotypes. Therfore, expanding the number of barley genotypes amenable to be improved through direct gene transfer.

SUMMARY

Engineering of novel traits in commercially important barley cultivars could play an important role in solving fundamental challenges. However, *in vitro* genetic manipulation of most commercial barley cultivars is still difficult because of the lack of an efficient regeneration system. Plant regeneration from immature embryos of four barley genotypes including two commercially important cultivars (Giza 123 and Giza 125) and two promising breeding lines (Line 9 and Line 11) was evaluated on four different MS-based culture media. The average number of embryogenic calli was improved for all genotypes by mainly decreasing the concentration of ferric sulfate, increasing the concentration of copper sulfate and boric acid and by substituting the 2, 4-D by Dicamba as growth regulator. EB medium revealed the highest frequency of embryogenic calli (182.8) and regenerated plantlets (16.13) across all genotypes. The highest regeneration frequency was exhibited by the genotype Giza 123 on EB medium (27.2%) followed by Giza 125 on the same medium (18.60%).

REFERENCES

Assem, S., K., E. H. A. Husseinand T. A. El-Akad (2008). Genetic transformation of Egyptian maize lines using the late embryogenesis abundant protein gene, HVA1, from barley. Arab J. Biotech., 11: 47-58.

Bregitzer, P. (1992) Plant regeneration and callus type in barley: Effect of genotype and culture media. Crop Sci., 32: 1108-1112.

Bregitzer, P., L. S. Dahleen and R. D. Campbell (1998). Enhancement of plant regeneration from embryogenic callus of commercial barley cultivars. Plant Cell Rep., 17: 941-945.

Cho, M. J., W. Jiang and P. G. Lemaux (1998). Transformation of recalcitrant cultivars through improvement in regenerability and decreased albinism. Plant Sci., 138: 229-244.

Dahleen, L. S. and P. Bregitzer (2002). An improved media system for high regeneration rates from barley immature embryo-derived callus of commercial cultivars. Crop Sci., 42: 934-938.

El-Itriby, H. A., S. K. Assem, E. H. A. Hussein, F. M. Abdel-Galil and M.

A. Madkour (2003). Regeneration and transformation of Egyptian maize inbred lines *via* immature embryo culture and biolistic particle delivery system. *In Vitro* Cell Dev. Biol., 39: 524-531.

FAO (1998). Production Yearbook, 52. Rom: FAO.

Fennell, S., N. Bohorova, M. Van Ginkel, L. Crossa and D. Hoisington (1996). Plant regeneration from immature embryo of 48 elite CIMMYT bread wheat. Theor Appl. Genet., 92: 163-169.

Hanzel, J. J., J. P. Miller, M. A. Brinkman and E. Fends (1985). Genotype and media effect on callus formation and regeneration of barley. Crop Sci., 25: 27-31.

Hussein, E. H. A., M. A. Madkour, S. K. Assem and R. Al-Zahraa R. (2004). Embryogencic callus formation and plant regeneration from immature embryos of some barley genotypes (*Hordeum vulgare* L.) Arab J. Biotech., 7: 111-122.

Lemaux, P. G., M. J. Cho, S. Zhang and P. Bregitzer (1999). Transgenic cereals: *Hordeum vulgare* L. (barley). In: Vasil IK (ed) Molecular Improvement of Cereal Crops. Kluwer, Dordrecht, p. 255-316.

Luthers, B. and H. Lörz (1987). Plant regeneration *in vitro* from embryogenic cultures of spring – and winter type barley (*Hordeum vulgare* L.) varieties. Theor. Appl. Genet., 75: 16-25.

Maddock, S. E. (1985). Cell culture, somatic embryogenesis and plant regeneration in wheat, barley, oats, rye and triticale. Martinus Nijhoff/ Dr. W. Junk Publisher, p. 131-174.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Tiidema, A. and E. Truve (2004). Efficient regeneration of fertile barley plants from callus culture of several Nordic cultivars. Hereditas, 140: 171-176.

Table (1): Components of the four media used for barley regeneration.

Basal salt medium(B.S)		Original medium(M1)		Improved medium(LD)		Modified medium(EB)	
Macro element		Macro element		Macro element		Macro element	
NH_4NO_3	20.6mM	NH_4NO_3	11.5mM	NH_4NO_3	11.5mM	NH_4NO_3	11.5mM
KNO_3	18.00mM	KNO_3	132mM	KNO_3	132mM	KNO_3	132mM
$CaCl_2$	3mM	$CaCl_2$	18mM	$CaCl_2$	18mM	$CaCl_2$	18mM
$MgSO_4.7H_2O$	1.5mM	$MgSO_4.7H_2O$	9.8mM	$MgSO_4.7H_2O$	9.8mM	$MgSO_4.7H_2O$	9.8mM
KH_2PO_4	1.25mM	KH_2PO_4	1.25mM	KH_2PO_4	1.25mM	KH_2PO_4	1.25mM
Microelements		Microelements		Microelements		Microelements	
H_3BO_3	0.1mM	H_3BO_3	0.1mM	H_3BO_3	0.75mM	H_3BO_3	0.75mM
$MnSO_4.4H_2O$	0.1mM	$MnSO_4.4H_2O$	0.1mM	$MnSO_4.4H_2O$	0.1mM	$MnSO_4.4H_2O$	0.1mM
KI	0.1mM	KI	0.005mM	KI	0.005mM	KI	0.005mM
$ZnSO_4.7H_2O$	0.03mM	$ZnSO_4.7H_2O$	0.03mM	$ZnSO_4.7H_2O$	0.03mM	$ZnSO_4.7H_2O$	0.03mM
$CoCL_2.6H2O$	0.2µM	$CoCL_2.6H2O$	1.0µM	$CoCL_2.6H2O$	1.0µM	$CoCL_2.6H2O$	1.0µM
$Na_2MoO_4.2H_2O$	1.0µM	$Na_2MoO_4.2H_2O$	1.0µM	$Na_2MoO_4.2H_2O$	1.0µM	$Na_2MoO_4.2H_2O$	1.0µM
$FeSO_4.{}_7H_2O$	0.1mM	$FeSO_4.{}_7H_2O$	0.1mM	$FeSO_4.{}_7H_2O$	0.05mM	$FeSO_4.{}_7H_2O$	0.05mM
$CuSO_4.5H_2O$	0.1µM	$CuSO_4.5H_2O$	0.1µM	$CuSO_4.5H_2O$	5.0µM	$CuSO_4.5H_2O$	5.0µM
Organic supplement		Organic supplement		Organic supplement		Organic supplement	
Myoinositol	100mg	Myoinositol	100mg	Myoinositol	100mg	Myoinositol	100mg
Thiamin-HCl	0.5mg	Thiamin-HCl	0.5mg	Thiamin-HCl	0.5mg	Thiamin-HCl	0.5mg
Solidifying agent		Solidifying agent		Solidifying agent		Solidifying agent	
Phytagel	3.5 gm	Phytagel	3.5 gm	Phytagel	3.5 gm	Phytagel	3.5 gm
Growth Regulators:		Growth Regulators:		Growth Regulators:		Growth Regulators:	
Initiation	3mg/L 2,4-D	Initiation	3mg/L 2,4-D	Initiation	3mg/L 2,4-D	Initiation	0.2mg/LDicamba
Maintenance	1.5mg/L 2,4-D	Maintenance	0.1mg/L BAP + 3mg/L 2,4-D	Maintenance	0.1mg/L BAP + 3mg/L 2,4-D	Maintenance	0.1mg/L BAP + 0.2mg/LDicamba
Regeneration	0.1 mg/L BAP	Regeneration	0.1 mg/L BAP	Regeneration	0.1 mg/L BAP	Regeneration	0.1 mg/L BAP
Rooting	none	Rooting	none	Rooting	none	Rooting	none
Carbon Source		Carbon Source		Carbon Source		Carbon Source	
Initiation	30 gm/L Sucrose	Initiation	30 gm/L Sucrose	Initiation	30 gm/L Maltose	Initiation	30 gm/L Maltose
Maintenance	30 gm/L Sucrose	Maintenance	30 gm/L Sucrose	Maintenance	30 gm/L Maltose	Maintenance	30 gm/L Maltose
Regeneration	30 gm/L Sucrose	Regeneration	30 gm/L Sucrose	Regeneration	30 gm/L Maltose	Regeneration	30 gm/L Maltose
Rooting	30 gm/L Sucrose	Rooting	30 gm/L Sucrose	Rooting	30 gm/L Sucrose	Rooting	30 gm/L Sucrose

Table (2): Mean number of induced embryogenic calli, maintained calli, regenerated plantlets and the regeneration frequency for the four tested barley genotypes (Giza 123, Giza 125, Line 9 and Line 11) on LD, EB, M1 and BS regeneration media.

Gepe	Medium	Mean number of embryogenic calli	Mean number of calli forming shoots	Mean number of regenerated plantlets	Regeneration frequency
Giza 123	LD	251.00[a]	221.50	20.00[b]	13.32
	EB	248.00[a]	220.00	41.00[a]	27.20
	M1	159.50[cd]	80.00	4.00[f]	2.60
	BS	35.50[g]	8.00	1.50[g]	1.00
Mean		173.50[a]	132.25	16.63[a]	11.03
Giza 125	LD	215.00[ab]	77.50	9.00[d]	12.00
	EB	162.50[cd]	111.00	14.00[c]	18.60
	M1	125.00[de]	59.00	4.00[f]	5.32
	BS	10.50[g]	0.00	0.00[g]	0.00
Mean		128.30[b]	61.87	6.75[b]	8.98
Line 9	LD	200.00[bc]	111.00	7.00[e]	9.32
	EB	210.00[ab]	17.00	1.00[g]	1.32
	M1	85.00[ef]	8.00	0.00[g]	0.00
	BS	6.50[g]	7.00	0.00[g]	0.00
Mean		125.30[b]	35.75	4.00[c]	2.6
Line 11	LD	48.00[fg]	14.00	7.00[e]	9.32
	EB	110.50[e]	23.00	9.00[d]	6.00
	M1	30.00[g]	0.00	0.00[g]	0.00
	BS	10.50[g]	0.00	0.00[g]	0.00
Mean		49.88[c]	9.25	1.88[d]	3.83

Table (3): Mean value of induced embryogenic calli and regenerated plantlets to represent the effect of medium on the ability of embryogenic callus formation and regenerated plantlets frequency from the four barley genotype.

Regeneration medium	Mean number of embryogenic calli	Mean number of regenerated plantlets
LD medium	178.8 [a]	10.75 [b]
EB medium	182.8 [a]	16.13 [a]
M1 medium	99.88 [b]	2.00 [c]
BS medium	15.75 [c]	0.3750 [d]

Table (4): Analysis of variance for the number of embryogenic calli and the number of regenerated plantlets.

Source of variation	DF	Mean square	
		Embryogenic calli	Regenerated plantlets
Genotype	3	21002.72*	340.21*
Medium	3	49760.37*	442.20*
Genotype x Medium	9	2870.98*	120.51*
Error	16	419.53	0.68

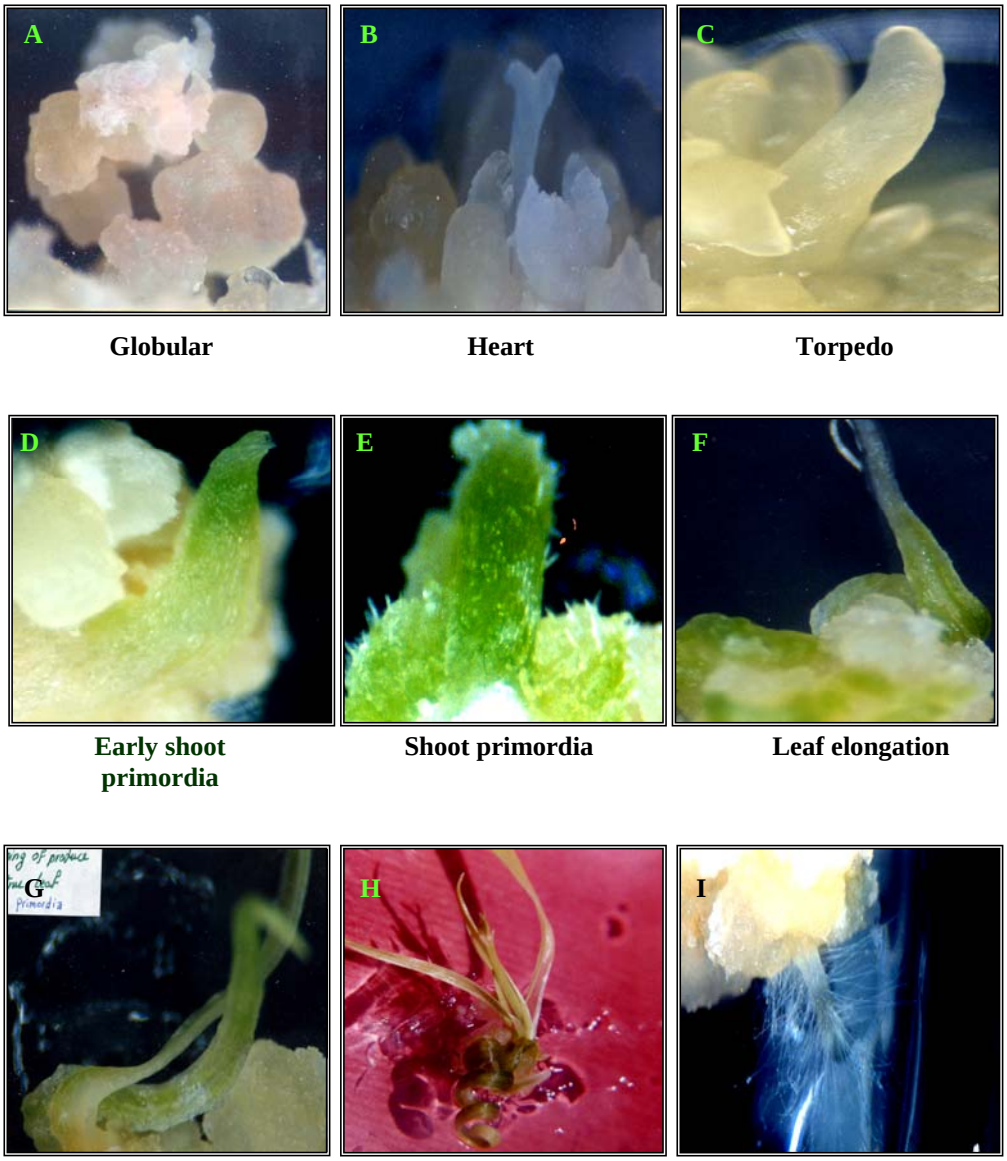

Fig. (2): Stages of somatic embryogenesis in barley as revealed by the cultivar Giza 123 on LD medium.

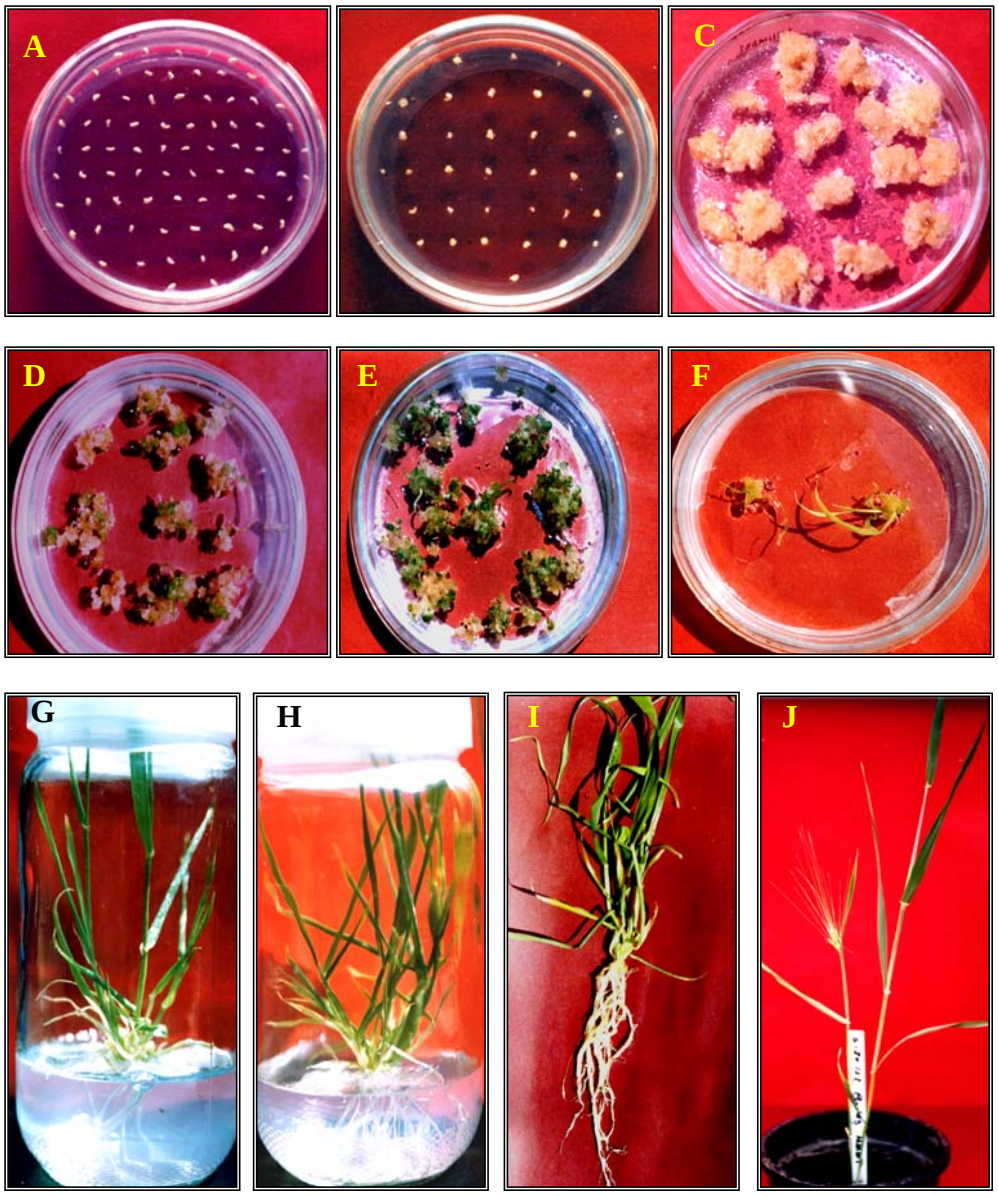

Fig. (2): Stages of barley regeneration of genotype Giza 123 on EB medium. (A) Bisected immature embryos cultured on callus induction medium, (B) Growing calli after 5 days, (C) 6 weeks old embryogenic calli, (D) Maintained calli, (E) and (F) Regenerated calli, (G) Well shooted plantlets on rooting medium after 1 weeks, (H) The same plantlets after 4 weeks, (I) The same plantlets prior transferring to soil, (J) Healthy and mature regenerated barley plants.