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Combined Effect of Gamma Radiation and Mannitol on Callus Formation and Regeneration in Alfalfa (*Medicago sativa* L.)

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ABSTRACT

Seed of two alfalfa cultivars Al-Wadi and Siwa were irradiated with γ radiation doses 0, 40, 80 and 120 Gy and shoot and leaf explants were cultured on MS medium supplemented with different NAA + 6 - BAP combinations and 8% or 9% mannitol. The cultures were incubated under light regimes for initiation and growth of callus and plant regeneration. With the increase γ-radiation dose and mannitol concentration, there were reductions in the callus growth and plant regeneration. Moreover, these characters were affected with light regimes and NAA + 6 - BAP combinations. The light was important factor for either callus growth or plant regeneration. The effects of NAA + 6 - BAP combinations were affected with other factors. The best combinations were 1.0 mg^{-1} NAA + 0.5 mg^{-1} BAP, 1.5 mg^{-1} NAA + 0.5 mg^{-1} BAP. The response of cv. Al-Wadi for callus formation and regeneration under these conditions was better than cv. Siwa. Regenerated plants under these conditions were abnormal, having short internodes, small and thicker greener leaves and less number of roots.

Key Words: Alfalfa; Light; Mannitol; Radiation; Somatic embryogenesis; Stress

INTRODUCTION

Broad applications of somatic embryogenesis, both in basic and applied research, have been stimulated studies on the determination of *in vitro* conditions for the induction of somatic embryos and their conversion into plants.

Studies on factors controlling *in vitro* plant morphogenesis are highly desirable not only for the development of improved regeneration systems, but also for the analysis of molecular mechanisms underlying plant embryogenesis (Gaj, 2004). Current progress in plant genetics and biotechnology is highly dependent upon the use of *in vitro* culture. Hence the establishment of effective *in vitro* plant regeneration systems leading to rapid production of fertile and, genetically pure plants is of great interest to plant biotechnologists.

Among the various *in vitro* systems applied, somatic embryogenesis is of special value. It offers opportunities for *in vitro* production of true to type plants by clonal propagation as well as regeneration of genetically modified plants by genetic transformation, somatic hybridization and *in vitro* mutant induction and selection (Gaj, 2004). Somatic embryogenesis is also induced directly from explants (primary somatic embryogenesis), or through callus (secondry somatic embryogenesis). Higher efficiency of secondary somatic embryogenesis over primary somatic embryogenesis has been reported for many plant species (Akula *et al*., 2000; Vasic *et al*., 2001). *In vitro* development of cells and tissues depends on different factors, such as genotype, type of plant, age and developmental stage of explant, physiological state of explant donor plant, and the external environment, which includes composition of media and physical culture conditions (light, temperature). Interaction between all these factors leads to the induction and expression of a specific mode of cell differentiation and development. Somatic embryos are formed by embryogenic cells, which arise from somatic cells of an explant, callus or suspension cells (De Jong *et al*., 1993).

It is widely recognized that somatic cells can acquire embryogenic potential as a result of different external chemical and physical stimuli, generally called stress factors (Maluszynski *et al*., 1996). Stress is commonly recognized as an essential component of embryogenesis induced in microspore culture – androgensis (Touraev *et al*., 1996 & Dunwell, 1996). However, a positive influence of stress was also observed in cultures of somatic tissue where development of somatic embryos was induced (Dudits *et al*., 1995). Embryogenic competence of *in vitro* cultured somatic cells can be stimulated by various factors, such as osmotic pressure, chlorides of heavy metals, pH, low or high temperature, starvation, mechanical wounding of explant or high auxin level (Kiyosue *et al*., 1993). Mutagens are also among stress factors that stimulate morphogenic process under *in vitro*. A stimulatory effect of physical and chemical mutgens on embryogenesis was reported in anther or microspore (Maluszynski *et al*., 1996).

The objective of this current study was to investigate the effect of γ-radiation doses, mannitol concentrations, genotypes, explanta, hormone combinations, light regime on callus induction and plant regeneration in alfalfa cvs. Al.-Wadi Al-Gadid and Siwa Tarkibi.

MATERIALS AND METHODS

Seed of two alfalfa (*Medicago sativa* L.) cultivars Al-Wadi Al-Gadid and Siwa Tarkibi, were obtained from Agriculture Research Center, Giza, Egypt.

Gamma radiation treatment. Seeds were irradiated with γ-rays with doses (0, 40, 80 or 120 Gy). ^{60}Co was used as a source of γ radiation with dose rate 1 k - rad/ 7.35 min, washed with tap water and surface sterilized by dipping in Clorox (30%) for ten min followed by three rinses in sterile distilled water. Seeds were cultured on MS medium (Murashige & Skoog, 1962) free hormones for germination. The seeds were divided to two parts; one incubated for germination under control light (L) 16/ 8 h and other part incubated for germination in dark (D). The pH of the culture medium was adjusted to 5.8 before autoclaving and incubated in growth chamber at 25ºC. Micro-cutting was made after 1 - 2 weeks when the plantlets were 4 cm high. Plantlets were cut to shoot and leaf explants and cultured on MS medium supplemented with different combinations of 1 - naphthalene acetic acid (NAA) and 6 - benzylamino-purine (6 - BAP) and 8% or 9% mannitol as fellow; 0.5 mg^{-1} NAA + 0.5 mg^{-1} BAP + 8% or 9% mannitol (M 1), 1.0 mg^{-1} NAA + 0.5 mg^{-1} BAP + 8% or 9% mannitol (M 2), 1.5 mg^{-1} NAA + 0.5 mg^{-1} BAP + 8% or 9% mannitol (M 3), 0.5 mg^{-1} NAA + 1.0 mg^{-1} BAP + 8% or 9% mannitol (M 4), and 0.5 mg^{-1} NAA + 1.5 mg^{-1} BAP + 8% or 9% mannitol (M 5).

Light regime. Plantlets produced in light (L) and dark (D) were cultured as shoot and leaf explants on M 1, M 2, M 3, M 4, or M 5 medium. The cultured jars incubated as follow; (1) cultured jars produced under light condition (L) were divided two parts, one part incubated under control light, light/ light (LL) and the other part incubated under dark condition, light/ dark (LD). Also, the cultured jars produced under dark condition (D) divided to parts, one part incubated under control light, dark/ light (DL) and the other part incubated under dark condition, dark/ dark (DD) for callus induction and plant regeneration.

Each treatment included 10 jars (120 x 60.5 mm) with 40 ml medium, containing 10 explants, for total 100 explants for each treatment.

The callus growth (g^{-1}) was evaluated after eight weeks.

RESULTS

Alfalfa plantlets cvs. Al-Wadi Al-Gadid and Siwa Tarkibi, produced from irradiated seeds with γ-radiation doses 40, 80 and 120 Gy were cutted to shoot and leaf explants and cultured on to MS medium supplemented with different NAA and 6 - BAP combinations and containing 8% or 9% mannitol concentrations. The cultured jars incubated under different light regime to initiation and growth of callus and plant regeneration.

Effect of γ-Radiation Doses 40, 80 or 120 Gy and 8 % Mannitol Concentration on Callus Induction Under Different Light Regimes and NAA + 6 BAP Combinations:

The effect of dose 40 Gy. Combined effects of γ radiation dose 40 Gy and 8% mannitol concentration on callus growth in cv. Al-Wadi, in shoot explant, the growth of callus was ranged between 0.0 g to 1.43 g. The highest callus growth was observed on M 3 at DL regime (Fig. 1). However, in leaf explant, the highest callus growth observed on M 4 at LL regime with weight 2.68 g. In cv. Siwa, on the other hand, the highest growth of callus was observed on M 3 under DD with weight 1.74 g in shoot explant. In leaf explant, the highest growth of callus observed on M 4 at DL regime with weight 1.6 g (Fig. 2).

The effect of dose 80 Gy. In cv. Al-Wadi, the highest growth of callus observed on M 3 and M 5 at LL regime with weight 1.2 g and 1.22 g, respectively in shoot explant. However, in leaf explant, the highest weight of callus observed on M 3 and M 5 with 1.03 g and 1.2 g, respectively (Fig. 3). In cv. Siwa, the highest growth of callus was observed on M 1 and M 3 under DL with 1.7 g and 1.77 g callus fresh weight, respectively and on M 3 at DD regime with 1.88 g callus in shoot explant (Fig. 4). However, in leaf explant, the highest callus growth was noted on M 3 and DL having 1.3 g weight of callus (Fig. 4).

The effect of dose 120 Gy and 8% mannitol on callus growth. In cv. Al-Wadi, the highest callus growth was observed on M 2 and LL regime and on M 3 and DL regime with weight 1.9 g and 1.15 g, respectively in shoot explant (Fig. 5). Moreover, in leaf explant, the highest callus growth was observed under LL and DL regimes on M 2 and M 4 with 1.5 g and 1.8 g fresh callus, respectively (Fig. 5). On the other hand, in cv Siwa the highest callus growth was noted on M 4 under LL and M 1 under DL regime with weight 0.88 g and 1.3 g, respectively in shoot explant (Fig. 6). However, in leaf explant, the highest growth of callus observed under LL and DL on M 5 and M 1 with 0.7 g and 1.05 g calli, respectively (Fig. 6).

The effect of 40, 80 and 120 Gy. γ-radiation doses and 9% mannitol concentration on callus growth. Shoot and leaf explant of cv. Al-Wadi exposed to 40 Gy and grown on MS medium containing 9% mannitol, the callus growth was the highest on M 1 and M 5 under LL and DL with 3.03 g and 2.5 g fresh weight, respectively in shoot explant (Fig. 7). However, in leaf explant, the highest callusing was observed under LL and DL regimes with 1.83 g and 2.67 g callus mass, respectively. Conversely, in cv. Siwa the callus growth was reduced under all treatments. In shoot explant, the callus growth on M 3 and M 4 was 1.83 g and 0.7 g, respectively under LL regime, but it failed to grow M 1, M 2 and M 5. However, it was 0.2 g and 0.6 g on M 2 and M 3, respectively under LD regime, but failed to form on M 1, M 4 and M 5. The callus weight was 0.53 g and 2.5 g on M 1 and M 2, respectively under DL but no callus formed on

Fig. 1. The combined effect of gamma radiation dose 40 Gy and 8% mannitol on callus growth in cv. Al-Wadi

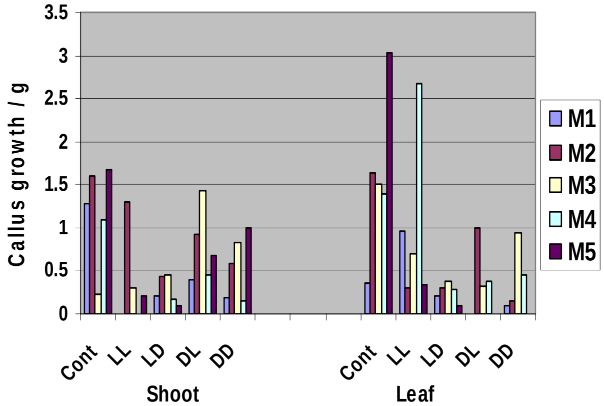


Fig. 2. The combined effect of gamma radiation dose 40 Gy and 8% mannitol on callus growth in cv. Siwa

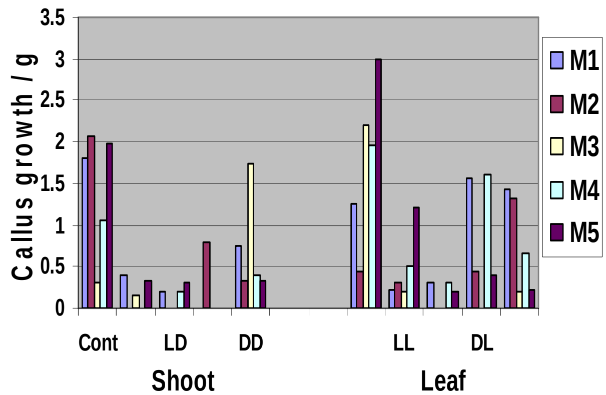
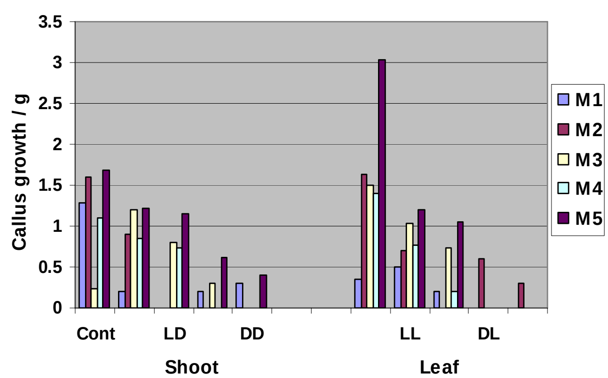


Fig. 3. The combined effect of gamma radiation dose 80 Gy and 8% mannitol on callus growth in cv.Al-Wadi



M 3, M 4 and M 5. There was no callus growth on M1, M2, M3, M4 and M5 under DD. In leaf explant, the callus growth under LL was 0.45, 1.73 and 0.4 g on M2, M3 and M4, respectively but none on M1 and M5. However, the callus could not grow under LD, DL and DD on M1, M2, M3, M4 and M5 except M2 under DL where it was 0.65 g.

Fig. 4. The combined effect of gamma radiation dose 80 Gy and 8% mannitol on callus growth in cv. Siwa

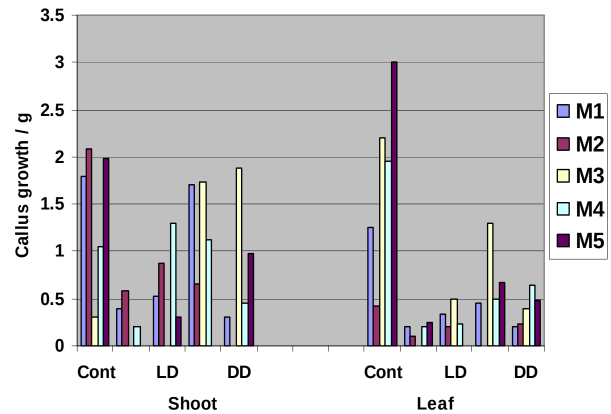


Fig. 5. The combined effect of gamma radiation dose 120 Gy and 8% mannitol on callus growth in cv. Al-Wadi

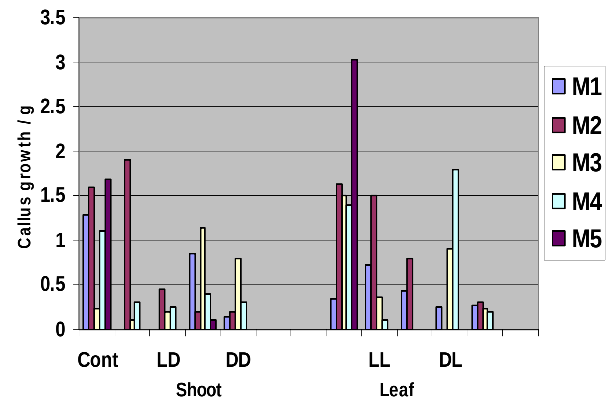
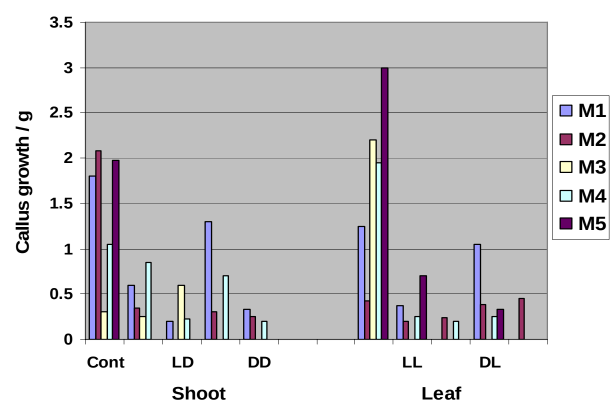


Fig. 6. The combined effect of gamma radiation dose 120 Gy and 8% mannitol on callus growth in cv. Siwa



The effect of 80 Gy and 9% mannitol concentration. In Cv. Ai-wadi, in shoot and leaf explants, the callus growth on M 1, M 2, M 3, M 4 and M 5 under LL was noted with 0.7 g, 0.2 g, 1.4 g, 0.7 g and 0.77 g mass, respectively in leaf explant. However, the calli failed to grow on most other treatments in both shoot and leaf explants except on M 3, M

4 and M 5 in shoot explant and M 1 and M 2 in leaf explant under LD with weight 0.33 g, 1.4 g and 0.1 g and 0.25 g, and 1.0 g callus mass, respectively and under DL on M 5 with weight 2.03 g in leaf explant. In cv. Siwa, these treatments were severe for shoot and leaf explants. Whereas, the shoot explant failed to form callus except on M 1, M 3 under LL and M 3 under LD with weight 0.3 g, 2.73 g and 0.3 g, respectively. However, in leaf explant the growth of callus was observed only on M 1, M 2, M 3, M 4 and M 5 under LL with 1.15 g, 0.25 g, 2.9 g, 0.2 g and 0.25 g callus mass, respectively and on M 3 and M 5 under LD with weight 1.47 g and 0.3 g per callus, respectively.

The effect of 120 Gy and 9% mannitol concentration. In shoot and leaf explants cvs. Al-Wadi and Siwa, the effect of these treatments were severe. In cv. Al-Wadi the callus formation observed in some treatments was; under LL on M 1, M 2 and under DL on M 1 with weight 0.23 g, 0.3 g and 0.2 g, respectively in shoot explant. But in leaf explant, the callus was observed on M 1 under LL with fresh weight 0.49 g and on M 1 and M 3 under LD with fresh weight 0.27 g and 0.43 g, respectively.

Gamma radiation and 8% or 9% mannitol concentrations effect on plant regeneration. The regeneration of shoot and leaf explants in both cultivars was affected negatively with combined effects of γ-radiation and mannitol concentrations. The effect of dose 40 Gy and 8% mannitol concentration on plant regeneration in shoot and leaf cv. Al-Wadi (Fig. 8). In shoot explant, the regeneration failed at most treatment except, under LL, the regeneration observed on M 2 and M 5 with ratio 8% for each. Also, the regeneration was observed on M 1 and M 2 under DL up to 28% and 33%, respectively and on M 1 and M 2 with ratio 17% and 8%, respectively under DD. However, in leaf explant, the regeneration was observed on M 2 and M 3 with ratio 8% for each under LL, also on M 2 and M 4 up to 55% and 43%, respectively under DL and on M 1 and M 2 under DD with ratio 17% and 8%, respectively. On the other side, in cv. Siwa both shoot and leaf explants were prone to these treatments (Fig. 9). The regeneration failed on most treatments. Except, under DD, the regeneration was observed on M 2, M 4 and M 5 with 8%, 8% and 10%, respectively. While in leaf explant, the regeneration observed on M 4 under DL with 1% and on M 1, M 2 and M 4 at DD regime with ratio 17% and 8%, respectively.

The effect of doses 80 and 120 Gy and 8% mannitol on plant regeneration. The regeneration was affected with these treatments whereas, it was severe on shoot and leaf explants in both cultivars. The regeneration percentage in shoot explant cv. Al-Wadi, which irradiated with 80 Gy was 7% and 17% on M 1 and M 4, respectively under LD, while regeneration failed with other treatments and the leaf explant. However, in cv. Siwa in both shoot and leaf explants, the regeneration failed except, on M 1 under LD up to 10%. The effect of 120 Gy on regeneration was also adverse. The regeneration in cv. Al-Wadi on M 2 under LL from shoot and leaf explants was 8% and 17%, respectively.

Fig. 7. The combined effect of gamma radiation dose 40 Gy and 9% mannitol on callus growth in cv. Al-Wadi

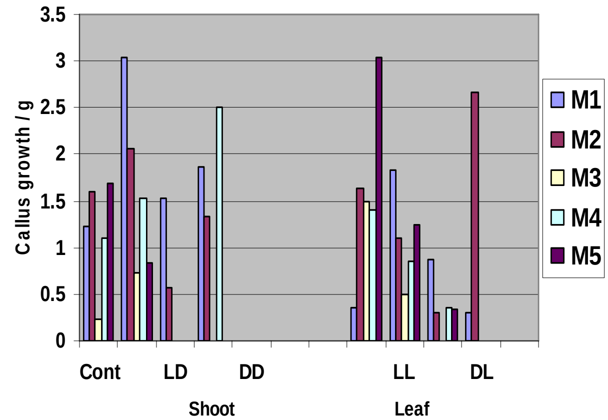


Fig. 8. The combined effects of dose 40 Gy and 8% mannitol on plant regeneration in alfalfa cv. Al-Wadi

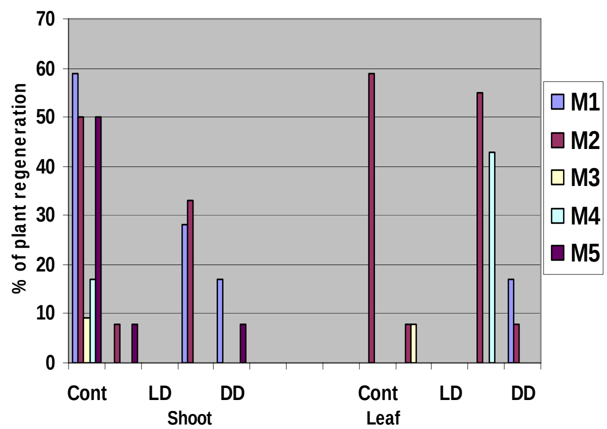
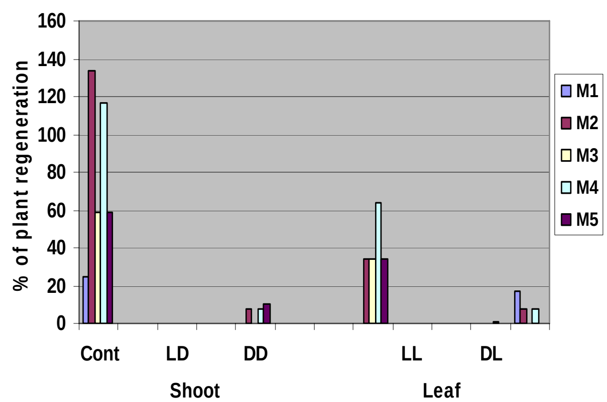


Fig. 9. The combined effects of dose 40 Gy and 8% mannitol on plant regenration in cv. Siwa



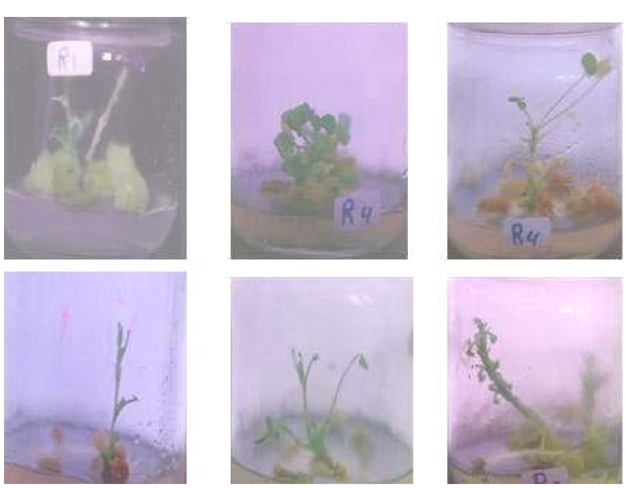
Moreover, in cv. Siwa the regeneration was noted only on M 2 at DD regime with ratio 17% in leaf explant.

Effect of γ-radiation doses 40, 80 and 120 Gy and 9% mannitol concentration on plant regeneration. Shoot and leaf explants in both cvs. Al-Wadi and Siwa were affected negatively. The regeneration failed at all treatments except some treatments. In shoot and leaf explants of cv. Al-Wadi irradiated with 40 Gy, the regeneration was observed only on M 1 and M 2 under LD with 33% and 8% in shoot and leaf explants, respectively. While the shoot and leaf irradiated with 80 Gy, the regeneration was noted only up to 10% on M 1 under DD in shoot explant and 5% on M 2 under LD in leaf explant. However, the effect of dose 120 Gy was severe on both cvs. Al-Wadi and Siwa. The regeneration failed in shoot and leaf explant and not formed either on M 1 under LL up to 6% in shoot explant cv. Al-Wadi. The regenerated plants under these conditions were, abnormal having shortened internodes small thicker greener leaves and reduce number of roots (Fig. 10).

DISCUSSION

The combined effects between γ-radiation doses, mannitol concentrations and NAA + 6 BAP on callus induction and regeneration under light regime in both shoot and leaf explants were variable. The highest growth of callus produced from irradiated shoot and leaf explants in cv. Al-Wadi were observed on M 3 and M 4 under DL and LL respectively, (Fig. 1). Moreover, in cv. Siwa, the highest callus fresh weight was observed also on M 3 and M 4 but under DD and DL, respectively (Fig. 2). The callus produced from irradiated shoot and leaf explants with 80 Gy and 8% mannitol, the highest callus growth observed on M 3 and M 5 in shoot explant and M 5 in leaf explant under LL in cv. Al-Wadi (Fig. 3). Also, in cv. Siwa, the highest callus fresh weights formed on M 3 in shoot explant under DD and on M 3 under DL in leaf explant (Fig. 4). Whereas, the highest callus fresh weight in shoot and leaf explants irradiared with 120 Gy and 8% mannitol were observed on M 2 and M 4 respectively, under LL and DL in cv. Al-Wadi (Fig. 5). In cv. Siwa, the highest callus formed on M 1 under DL in both shoot and leaf explants (Fig. 6). The increasing mannitol concentration to 9% with γ-radiation doses was affected negatively on callus formation and callus fresh weight. The highest callus fresh weight formed on M 1 under LL in shoot explant and M 2 under DL in leaf explant cv. Al-Wadi (Fig. 7). In cv. Siwa, the highest callus fresh weight observed on M 2 under DL in shoot explant and on M 3 under LL, which irradiated with 120 Gy and 9% mannitol. The effect of 120 Gy and 9% mannitol were severe on callus formation in cv. Siwa. The increasing γ-radiation doses 80 Gy and 9% mannitol were severe on callus formation in both cultivars. Whereas, the callus formation failed completely in cv. Siwa, which irradiated with 120 Gy. Moreover, the ratio of regeneration was affected with γ-radiation and mannitol concentrations (Fig. 8 & 9). The type of explant and its well defined developmental stage seams to be the most important factor, which determines the embryogenic capacity of the culture.

Fig. 10. The effect of stress conditions, radiation, mannitol and light on regenerated plants in alfalfa cvs. Al-Wadi and Siwa



Somatic embryogenesis can be induced in cultures of various explant types as seedling and their fragments, petioles, leaves, shoot meristems, roots, seeds, cotyledons and zygotic embryos (Raemakers *et al*., 1995). Different types of explant used to induce somatic embryogenesis displayed the highest response at a certain age, as was documented in the culture of cotyledons of *Helianthus annuus* (Fiore *et al*., 1997), leaves of *Archis hypogaea* (Baker & Wetzstein, 1998) and seeds of *Brassica napus* (Koh & Loh, 2000). The level of endogenous phytohormones is considered as one of the crucial factors influencing embryogenic potential of explant. The response of explants to embryogenic transition undergo accumulation of endogenous auxin (IAA) rather than display stable, high level of endogenous hormones. Such increase in IAA level in the tissue during the first days of culture under embryogenic conditions was observed in carrot cells (Michalczuk *et al*., 1992) alfalfa protoplasts (Pasternak *et al*., 2002) and immature zygotic sunflowers embryos (Charriere *et al*., 1999). Thus, auxin synthesis induced by embryogenic conditions is supposed to be one of the crucial signals determining embryogenic fate of culture cell (Thomas *et al*., 2002). In culture of alfalfa protoplasts a delay of few days in the synthesis of IAA was indicated in non-embryogenic cells in comparison to embryogenic ones (Pasternak *et al*., 2002). The influence of light on *in vitro* plant morphogenesis can be related with stimulatory or inhibitory effect of light on different endogenous substances, including plant growth regulators (Zelena, 2000). Photolysis of endogenous plant growth regulators under high light, and its detrimental effect on *Dianthus*, *Crataegus* and *Rhododendrom* shoots developing *in vitro* were observed by Marks and Simpson (1999).

Molecular mechanisms involved in light mediated stimulation or inhibition of somatic embryogenesis awaited determination. Analysis of somatic embryogenesis induction in immature zygotic embryos of wheat has indicated light modulated expression of some proteins involved in this process (Nato *et al*., 2000). Among molecules that play a key role in light stimulation of gene expression are phytochromes. These plants photoreceptors interact with transcription factors and regulate the rxpression of numerous genes and the molecular mechanisms by which phytochromes transduced light signals have been elucidated (Kevei & Nagy, 2003). In tomato cultures explants, light dependent acquisition of competence for shoot formation mediated by phytochromes was described (Bertram & Lercari, 2000). It has become widely recognized that somatic cells can aquire embryogenic potential as a results of different external chemical and physical stimuli, generally called stress factors. Osmotic pressure applied in an induction medium was reported to stimulate somatic embryogenesis in explants of *P. ginseng* (Choi *et al*., 1998) and *C. sinensis* (Akula *et al*., 2000). Stress factors that can stimulate morphogenic process under *in vitro* conditions induce also mutagens. A stimulatory effect of physical and chemical mutagens on embryogenesis was reported in anther or microspore culture (Maluszynski *et al*., 1996) and in somatic tissues cultures. An increase in shoot multiplication of *Musa* ssp. Due to γ-irradiation was described by Kulkarni *et al.* (1997). Similarly, γ-radiation stimulated plant regeneration in cultures of *Eleusine coranna* embryogeneic calli (Pius *et al*., 1994) and improved shoot organogenesis and somatic embryogenesis in culture of *H. annuus* immature zygotes embryos (Encheva *et al*., 1993). All of the stress factors induce a common reaction of somatic cells manifested by their de- and re- differentiation to somatic embryos. However, the stimulatory effect of stress treatment on cell differentiation and morphogenesis remain elusive. The induction of abnormal cell divisions (MacDonald & Aslam, 1986; Zaki & Dickinson, 1995), accumulation of endogenous auxins (Zimmerman, 1993), changes in the cytoskeleton components (Iqbal *et al*., 1994), or the induction of stress protein (Rey *et al*., 2002), were thought to explain the mode of stress factor activity. Whatever the detail of mechanisms is, stress treatment triggers expression of factor (s), which effect gene expression and cell cycle regulation and thus induce somatic embryogenesis. Identified factors controlling stress specific response genes (Aarts & Fiers, 2003) can help to elucidate the stress response in plants and its relation to embryogenesis. Whereas, some treatments enhanced callus growth, regeneration, other enhanced callus growth only and some of them inhibited both callus growth and regeneration. The increasing γ-radiation doses and mannitol concentration induced decreasing in callus fresh weight and regeneration percentage. Callus fresh weight and regeneration in shoot explant was better than leaf explant. The best combination for callus growth was 1.5 mg^{-l} NAA + 0.5 mg^{-l} BAP and 1.0 mg^{-l} NAA+ 0.5 mg^{-l} BAP for plant regeneration. Al-Wadi cultivar was more resistance than Siwa cultivar. The regenerated plants under these conditions were abnormal having shortened internodes small thicker greener leaves and reduce number of roots (Figs. 10). These results are in accordance with those obtained with tomato (Locy, 1995) and sweet potato (El-Fiki, 2004).

CONCLUSION

Response of alfalfa shoot and leaf explants cvs. Al-Wadi and Siwa produced from seed irradiated seeds with γ-rays for callus induction and plant regeneration dependeds on genotype, explant type, hormone concentrations and their combinations, light, mannitol concentration and γ-radiation dose. Some treatments can be used to induced callusing and regeneration and others for inhibition for all. These stress factors induced common reaction of somatic cells manifested by their de – and re – differentiation to somatic embryos.

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