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Fast versatile regeneration of *Trifolium alexandrinum* L.

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Abstract *Trifolium alexandrinum* L. (Egyptian clover) is one of the most important forage crops in the world. Its regeneration in tissue culture has been described in a few reports but the efficiency, accurate time scales and applicability to various genotypes of the described procedures are uncertain. Therefore their suitability for genetic transformation is unclear. In this study, were report new fast procedures for regeneration of Egyptian clover that are applicable to the regeneration of various genotypes (Mescawi-ahaly, Sakha3 and Sakha4). Shoots were regenerated from intact and wounded cotyledons as well as hypocotyls of Mescawi-ahaly on naphthaleneacetic acid/benzyladenine (NAA/BA) and naphthaleneacetic acid/thidiazuron (NAA/TDZ) media. The highest shoot regeneration frequencies were obtained from intact cotyledons on NAA/BA (0.05 mg l^{-1} NAA combined with 2.0 mg l^{-1} BA) and NAA/TDZ (0.05 mg l^{-1} NAA combined with 1.0 mg l^{-1} TDZ) media (66.2 and 43.1% respectively) compared to 18.4 and 10.1% for wounded cotyledons on NAA/BA and NAA/TDZ respectively. 21.0% shoot regeneration frequency was observed for hypocotyls on NAA/BA (2.0 mg l^{-1} NAA combined with 0.5 mg l^{-1} BA) medium but no regeneration was obtained on NAA/TDZ medium. Rooting of the regenerated shoots was induced on indole butyric acid (IBA: 0.24 mg l^{-1}) or NAA (2.0 mg l^{-1}) media where IBA medium supported significantly higher frequencies of rooting as well as survival of the whole plantlets after transfer to soil. However, the rooting and survival frequencies also depended on the type of explant and the medium used for shoot regeneration. The two cultivars Sakha3 and Sakha4 were regenerated using the culture conditions optimized for Mescawi-ahaly with comparable efficiencies, indicating that the described procedure is not genotype dependent. The time scale of whole plantlet regeneration ranged from 7.5 weeks for intact and wounded cotyledons to 10 weeks for hypocotyl explants.

Keywords *Trifolium alexandrinum* · Genotype independent regeneration · Shooting media · Rooting media

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Introduction

Egyptian clover (*Trifolium alexandrinum* L.) is a very important annual nitrogen-fixing leguminous crop. It is grown in the winter for forage in the Mediterranean basin, the Indian subcontinent, central Asia and parts of the United States (Knight 1985; Roy et al. 2005). In Egypt, it is the only forage crop grown during the winter. It is also an essential component of the crop rotation where it must be grown at least once every 2 years to enrich the soil with nitrogen prior to cultivation of summer crops such as cotton and rice.

Efficient methods for plant regeneration via tissue culture are essential for crop improvement by gene transformation. Very few reports have described the regeneration of Egyptian clover in tissue culture (Mokhtarzadeh and Constantin 1978; Barakat 1990). However, in these reports the regeneration was genotype dependent. In addition, no accurate time scales were included for the regeneration process. Tanaka et al. (2001) reported the regeneration of Egyptian clover from roots after *Agrobacterium rhizogenes* transformation. Only one genotype (Mescawi-ahaly) was

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used in this study and the regeneration required several months to complete.

However, methods for tissue culture regeneration of other *Trifolium* species have been described in many reports. An efficient method has been reported for regeneration of white clover (*Trifolium repens* L.) from cotyledons where the regenerated shoots were fully developed and ready to transfer to soil after 6–8 weeks from culture initiation (White and Voisey 1994). Tissue culture regeneration of subterranean clover (*Trifolium subterraneum* L.) after *Agrobacterium tumefaciens* -mediated transformation has been described (Khan et al. 1994). A procedure for *A. tumefaciens*-mediated transformation and regeneration has also been described for red clover (*Trifolium pretense* L.; Quesenberry et al. 1996). A comprehensive report has described an efficient method for plant regeneration after *A. tumefaciens*-mediated transformation of five *Medicago* and five *Trifolium* species not including Egyptian clover (Ding et al. 2003).

In spite of the economic importance of Egyptian clover, its genetic improvement by *A. tumefaciens* -mediated transformation has never been reported. This could be due to the lack of a fast genotype independent method for its tissue culture regeneration. This highlights the need for efficient methods for regeneration of this species. In this report, we describe new fast methods for regeneration of Egyptian clover using different types of explants and compare these methods to the previously described ones. We also test the effects of different combinations of plant growth regulators on the regeneration process.

Materials and methods

The landrace Mescawi-ahaly (which is maintained by local farmers in the Egyptian Delta), in addition to two cultivars of Egyptian clover (Sakha3, Sakha4) were used in this study. Sakha3 and Sakha4 are produced and maintained by the Agricultural Research Centre in Egypt. They are important cultivars of Egyptian clover due to their high productivity since they give 4–6 harvests per cultivation (Badr et al. 2008). The regeneration conditions were optimized for Mescawi-ahaly and the optimized conditions were tested for regeneration of Sakha3 and Sakha4.

Seed germination and seedling growth

The seeds were surface-sterilized with 70% ethanol for 1 min and then with 0.1% mercuric chloride containing 0.01% Tween 20 for 10 min. They were then washed with sterile water (at least five changes) for 15 min. The sterile seeds were germinated on MS basal medium (Murashige and Skoog 1962) without sucrose and solidified with 0.6% agar in 175 ml tissue culture vessels (Sigma–Aldrich). The seeds were incubated at 25°C, 12 h photoperiod and 100 µmole m^{-2} s^{-1} light intensity in Sanyo MLR-350 incubator (Sanyo electric company, Japan). All subsequent cultures were incubated under the same conditions unless specified.

Shoot regeneration from intact cotyledon explants

Cotyledons from 3 days old seedlings were dissected aseptically under water by cutting the cotyledons with 1–2 mm of the cotyledon stalk attached (Fig. 1a, b). The shoot tips (if present) were discarded. The cotyledons were then planted with adaxial sides up onto MS basal medium supplemented with 2% sucrose and different combinations of naphthaleneacetic acid (NAA: 0.0, 0.05, 0.1, 0.5, 1.0 or 2.0 mg l^{-1}) and benzyladenine (BA: 0.5, 1.0, 2.0 or 4.0 mg l^{-1}) and solidified with 0.7% agar in Petri dishes (NAA/BA medium). In another experiment, thidiazuron (TDZ: 0.5, 1.0, or 2.0 mg l^{-1}) was used instead of BA combined with the previous NAA concentrations (NAA/TDZ medium). The pH of all media was adjusted to 5.75 with 1% KOH prior to autoclaving. Six Petri dishes were used for each NAA/cytokinin combination and each Petri dish contained 20 explants. The cultures were incubated in the dark for 5 days (by wrapping them with tin foil) at 25°C in Sanyo MLR-350 incubator (Sanyo electric company, Japan). The foil was then removed and the cultures were exposed to 100 µmole m^{-2} s^{-1} light intensity up to the end of experiment. After 3 weeks, the regenerating explants were counted and subcultured onto MS basal medium supplemented with 2% sucrose, 0.05 mg l^{-1} NAA and 0.1 mg l^{-1} BA and solidified with 0.7% agar (shoot development medium: SD) and allowed for 10 days for further shoot development.

Shoot regeneration from wounded cotyledon explants

Cotyledon explants were dissected aseptically from 3 days old seedlings as before but they were wounded by making a lengthwise cut in the distal half of the each cotyledon (Fig. 1f, g). The wounded cotyledons were replicated and cultured as described for intact cotyledons.

Shoot regeneration from hypocotyl explants

Hypocotyls were dissected from 4 days old seedlings by removing the cotyledons at the hypocotyl nodes along with the roots. The remaining hypocotyls (about 1 cm long) were used as explants (Fig. 1j, k). The hypocotyls were planted horizontally onto MS medium (pH 5.75) supplemented with 2% sucrose and different combinations of NAA (0.5, 1.0, 2.0 or 5.0 mg l^{-1}) and BA (0.1, 0.5, 1.0 or

Fig. 1 Steps in the regeneration of Mescawi-ahaly clover from intact cotyledons (**a**–**e**), wounded cotyledons (**f**–**i**) and hypocotyls (**j**–**n**). **a** 3 days old seedling from which intact cotyledons were dissected by cutting at the *arrow*. **b** Dissected cotyledons immediately before culturing. **c** Small shoots regenerated on the cotyledon stalks after 2 weeks from culture initiation. d Same as **c** but at higher magnification. **e** Fully developed shoots after 4.5 weeks from culture initiation. **f** Cotyledons were obtained by cutting at the arrow. **g** Wounded cotyledons after 3 days of culturing. *h* Shoot regeneration on wounded cotyledons after 3 weeks of culturing. **i** Fully developed shoots after 4.5 weeks and immediately before subculturing onto rooting medium. **j** The 5 days old seedling used as source of hypocotyl explants, *arrows* delimit the part of seedling used as explant. **k** Isolated hypocotyl explant. **l** Small shoots developing on hypocotyls explant after 4 weeks of culturing. **m** Fully developed shoots after 6 weeks from culture initiation. **n** As in **m** but shoots separated

2.0 mg l^{-1}) and solidified with 0.7% agar in Petri dishes. TDZ (0.1, 0.5, 1.0 or 2.0 mg l^{-1}) combined with the previous concentrations of NAA was also tested in a separate experiment. Six Petri dishes were used for each NAA/cytokinin combination and each Petri dish contained 20 explants. The cultures were incubated in the dark for 5 days and then exposed to light as described before. They were subcultured after 2 weeks onto the same medium composition for further 2 weeks. The regenerating hypocotyls were then counted and subcultured onto SD medium for 2 weeks.

Rooting of the regenerated shoots

Shoots with proper stems (1–3 cm long) and trifoliate leaves were rooted on MS basal medium supplemented with 1.5% sucrose and either 0.24 mg l^{-1} indole butyric acid (IBA; Beattie and Garrett 1995) or 2.0 mg l^{-1} NAA (Barakat et al. 1990) in 175 ml tissue culture vessels (Sigma–Aldrich). The shoots were kept on the same medium for 3–4 weeks without any subculturing until they developed roots and were then transferred to soil. For each shoot regeneration medium, six rooting replicates (vessels) were used. Each replicate contained eight shoots.

Transfer of plantlets to soil

Prior to transfer to soil, the plantlets were kept in the dark for 2 h. They were then transferred to 6 cm pots containing compost (one plantlet per pot) and acclimated at high humidity for 2 days before exposed to growth room conditions (25°C, 60% RH, 12 h photoperiod and 200 µmole

m^{-2} s^{-1} light intensity). The survival of plantlets was evaluated 10 days after the transfer to growth room by counting the surviving and failing plantlets. Plantlets that failed to produce new leaves were considered failing even if they did not die.

Statistical analysis

To compare between different treatments (explant/phytohormone) in terms of shoot regeneration, root induction or plant survival, one way ANOVA was performed with SPSS version 12.0.1. For each treatment, the numbers of regenerating explants, rooting shoots or surviving plants were expressed as percentage for each replicate and introduced into SPSS 12.0.1. All comparisons were performed at significance level of $P < 0.05$.

Results

Regeneration of the landrace Mescawi-ahaly

Preliminary experiments for shoot regeneration from all types of explants showed that exposure to light in the first 5 days of culturing resulted in extensive browning and shrinkage of the explants particularly at the cut ends. This caused large proportions of the explants to die or at least cease growing. To avoid this, all cultures were kept in the dark immediately after culturing for 5 days.

Shoot regeneration from intact cotyledons

After 10 days of culturing of the intact cotyledons on NAA/BA medium, the cotyledon stalks swelled and showed meristemoids on their adaxial sides that developed into small shoots after further 4 days (Fig. 1c, d). Shoots with trifoliate leaves were observed after 3 weeks of culturing. These shoots were subcultured onto SD medium for further development. After 10 days, the shoots developed several fully expanded trifoliate leaves and were then subcultured onto the rooting medium (Fig. 1e). This response was not observed on the abaxial side even when the cotyledons were cultured with abaxial sides up. The cotyledon blades enlarged in the first few days of culturing but no further responses were observed except at 2 mg l^{-1} NAA combined with lower BA concentrations (0.5 and 1.0 mg l^{-1}) where small calli and roots developed after 3 weeks of culturing. Analogous shoot regeneration from the cotyledon stalks as well as responses of the cotyledon blades were observed on NAA/TDZ medium. However, the regenerated shoots did not grow as fast as these regenerated on NAA/BA medium (data not shown).

The highest frequency of shoot regeneration on NAA/BA medium was observed at 0.05 mg l^{-1} NAA combined with 2.0 mg l^{-1} BA where 66.2% of the intact cotyledons regenerated shoots (Fig. 2a). Concentrations of NAA lower or higher than 0.05 mg l^{-1} resulted in significant decreases of shoot regeneration frequencies. At lower concentrations of NAA (0.0–0.5 mg l^{-1}), the regeneration frequency increased significantly with increasing BA concentration up to 2.0 mg l^{-1} and then decreased at 4.0 mg l^{-1} BA. At 1.0 mg l^{-1} NAA, the highest shoot regeneration frequencies were observed at 0.5 and 1.0 mg l^{-1} BA (15.3 and 18.1% respectively). Higher concentrations of BA resulted in significant decrease of regeneration frequency. At 2.0 mg l^{-1} NAA, the highest regeneration frequency was found at 0.5 mg l^{-1} BA and higher concentrations of BA significantly decreased the regeneration frequency where no regeneration at all was obtained at 4.0 mg l^{-1} BA. On NAA/TDZ medium, the highest shoot regeneration frequency (43.1%) was found at 0.05 mg l^{-1} NAA combined with 1.0 mg l^{-1} TDZ (Fig. 2b). The regeneration frequencies decreased significantly at NAA concentrations lower or higher than 0.05 mg l^{-1}. At all NAA concentrations, 1.0 mg l^{-1} TDZ gave significantly higher frequencies of shoot regeneration compared to 0.5 and 2.0 mg l^{-1} except at 0.0 and 1.0 mg l^{-1} NAA where 2.0 mg l^{-1} TDZ resulted in the same shoot regeneration frequency as 1.0 mg l^{-1}. It was not possible to count the shoot numbers per explant due to the large numbers of shoots particularly at 1.0 and 2.0 mg l^{-1} BA and 1.0 mg l^{-1} TDZ combined with 0.05 mg l^{-1} NAA, where up to 15 shoots were clearly seen on each cotyledon stalk in addition to several small shoots.

Shoot regeneration from wounded cotyledons

The wounded cotyledons enlarged over the first few days of culturing (Fig. 1g) and showed meristemoid areas at the cut edges of their blades. Shoots were visible at the cut edges of the cotyledon blades after 3 weeks of culturing (Fig. 1h). The shoots originated from the abaxial side of the cotyledon blade (facing the nutrient medium). In most regenerating wounded cotyledons, no regeneration occurred from the cotyledon stalks. The shoots were subcultured onto SD medium for 10 before being subcultured onto rooting medium (Fig. 1i). The numbers of shoots on each wounded cotyledons ranged from one to four. Direct root regeneration from the wounded cotyledons was observed at 2.0 mg l^{-1} NAA combined with 0.5 or 1.0 mg l^{-1} BA. Similar shoot regeneration was observed in NAA/TDZ media but the shoots were smaller. Some shoots showed necrotic leaf edges after subculturing to SD medium and did not develop normally.

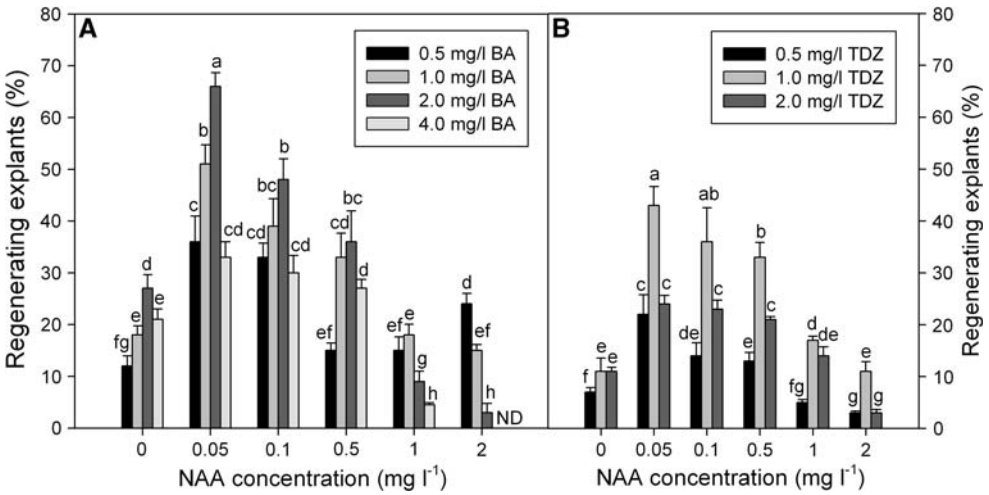

Fig. 2 Shoot regeneration from intact cotyledons on NAA/BA medium (**a**) and NAA/TDZ medium (**b**). *Bars* are the average frequencies of regeneration from six replicates (%) plus or minus SE. Counts were taken 3 weeks after culture initiation. In each separate graph, *bars* not sharing the same *small letter* labels are significantly different at $P < 0.5$. Statistics was performed for each graph separately due to the large numbers of treatments. ND on the *x*-axis of A means not detectable

The highest frequency of shoot regeneration by wounded cotyledons on NAA/BA medium (18.4%) was observed at 0.05 mg l^{-1} NAA combined with 2.0 mg l^{-1} BA (Fig. 3a). Concentrations of NAA lower or higher than 0.05 mg l^{-1} resulted in significant decreases in the regeneration frequency. At all NAA concentrations, the highest frequencies of shoot regeneration were observed at 2.0 mg l^{-1} BA except at 0.0 and 1.0 mg l^{-1} NAA where the regeneration frequency was significantly higher in the presence of 1.0 mg l^{-1} BA. No shoots were regenerated at 2.0 mg l^{-1} NAA combined with 0.5 or 4.0 mg l^{-1} BA. On NAA/TDZ medium, the highest frequency of shoot regeneration (10.1%) was observed at 0.05 mg l^{-1} NAA combined with 1.0 mg l^{-1} TDZ (Fig. 3b). Similar to NAA/BA medium, the optimum NAA concentration in NAA/TDZ medium was 0.05 mg l^{-1}. At all NAA concentrations, the highest frequencies of shoot regeneration were observed at 1.0 mg l^{-1} TDZ except at 0.0 mg l^{-1} NAA where 2.0 mg l^{-1} TDZ gave similar regeneration frequencies. No shoot regeneration was found at 0.5 mg l^{-1} TDZ combined with 0.0, 0.5, 1.0 or 2.0 mg l^{-1} NAA. Moreover, no shoots were regenerated at 2.0 mg l^{-1} TDZ combined with 1.0 or 2.0 mg l^{-1} NAA.

Shoot regeneration from hypocotyls

The hypocotyl explants developed calli at their proximal cut ends (root sides) after 4 weeks of culturing (Fig. 1l). These calli never produced shoots. Contrarily, the distal ends did not develop visible calli but gave rise to multiple shoots ranging in number from 2 to 5. The shoots were suitable for subculturing onto rooting media after further 2 weeks on SD medium (Fig. 1m, n).

Shoot regeneration was observed at 2.0 and 5.0 but not at 0.5 or 1.0 mg l^{-1} NAA (Fig. 4). The frequency of shoot regeneration at 2.0 and 5.0 mg l^{-1} NAA depended on BA concentrations. At 2.0 mg l^{-1} NAA, the regeneration frequency increased significantly with increasing BA concentrations where it reached its highest value (21.0%) at 1.0 mg l^{-1} BA and then decreased at 2.0 mg l^{-1} BA. At 5.0 mg l^{-1} NAA, no shoots were regenerated in the presence of 0.1 or 0.5 mg l^{-1} BA. However, low frequencies of shoot regeneration were observed in the presence of 1.0 and 2.0 mg l^{-1} BA (2.3% and 4.6% respectively). No shoot regeneration from hypocotyls was observed on any NAA/TDZ combination. Most hypocotyls accumulated purple pigmentations after 2 weeks from culture initiation and did not show any further development over the next 2 weeks.

Rooting of the regenerated shoots

Root induction was performed on media containing 0.24 mg l^{-1} IBA or 2 mg l^{-1} NAA. On IBA medium, the roots were visible after 6 days of subculturing onto the rooting medium. After further 2 weeks, the roots appeared well-developed and suitable for transplanting to soil. Incubation of the rooted shoots (that were regenerated from intact or wounded cotyledons) on the rooting medium for more than 3 weeks resulted in extensive branching and elongation of the roots that made them difficult to remove from the medium. However, shoots developed from hypocotyls needed up to 4 weeks on IBA medium for proper root development. On NAA medium, the roots were visible after 2 weeks of subculturing onto the rooting medium. All shoots were allowed for further 2 weeks for

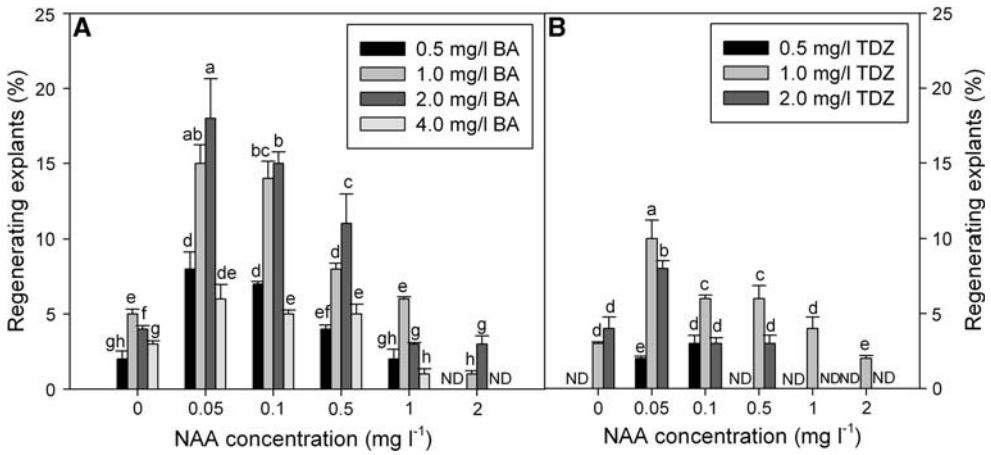

Fig. 3 Shoot regeneration from wounded cotyledons on NAA/BA medium (**a**) and NAA/TDZ medium (**b**). *Bars* are the average frequencies of regeneration from six replicates (%) plus or minus SE. Counts were taken 3 weeks after culture initiation. In each separate graph, *bars* not sharing the same *small letter* labels are significantly different at $P < 0.5$. Statistics was performed for each graph separately due to the large numbers of treatments. ND on the *x*-axis of **a** and **b** means not detectable

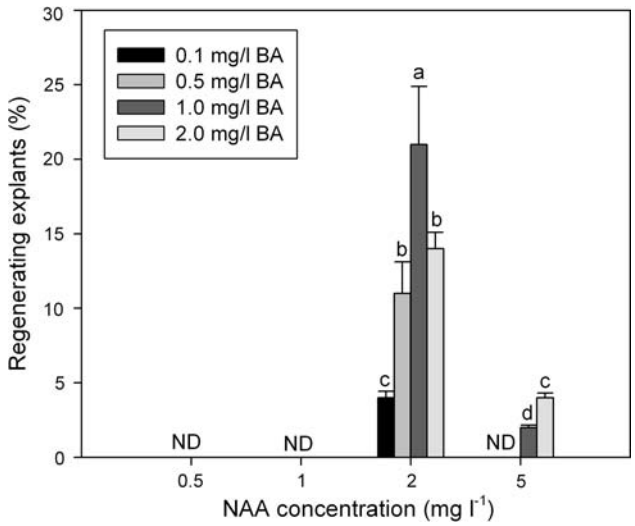

Fig. 4 Shoot regeneration from hypocotyls on NAA/BA medium. *Bars* are the average frequencies of regeneration from six replicates (%) plus or minus SE. Counts were taken 4 weeks after culture initiation. *Bars* labeled with different *small letters* are significantly different at $P < 0.5$. ND on the *x*-axis means not detectable. Data for NAA/TDZ medium is not presented because no regeneration was detected on this medium

root development. However, the roots appeared swelled and some of them developed from the calli (which were formed after subculturing onto the rooting medium) rather than from the base of stem. The rooted shoots were then transplanted to pots containing compost and were acclimated at high RH before transferred to growth room at 25°C, 12 h photoperiod, 60% RH and 200 μmole m^{-2} s^{-1}.

On IBA rooting medium, the frequency of root induction varied depending on the cytokinin and the type of explant (origin of the shoot) used for shoot regeneration (Fig. 5a). Shoots regenerated on NAA/BA medium gave significantly higher (up to tenfolds) frequencies of rooting than these regenerated on NAA/TDZ medium. The rooting frequencies of shoot regenerated on NAA/BA medium ranged from 21.8% in shoots regenerated from hypocotyls to 65.5% in these regenerated from intact cotyledons. No significant differences were observed in the rooting frequencies between shoots regenerated from intact and wounded cotyledons on NAA/TDZ medium (6.0 and 8.4% respectively; hypocotyls did not regenerate shoots on this medium).

On NAA rooting medium, the rooting frequencies followed the same trends as these on IBA medium except that the shoot regenerated from intact and wounded cotyledons on NAA/TDZ medium gave similar rooting frequency (Fig. 5b). However, the rooting frequencies on NAA medium were significantly lower than these on IBA regardless of the shoot origin or the cytokinin used for shoot regeneration except for shoots regenerated from hypocotyls which showed similar rooting frequencies on IBA and NAA media.

Survival of the shoots transferred to soil

Survival of the shoots after transfer to soil varied largely according to the rooting medium (Fig. 6a, b). However, it also depended on the shoot origin and the shoot regeneration medium. In all cases, the plantlet survival frequencies were significantly higher for the shoots rooted on IBA medium than on NAA medium. Most of the shoots regenerated from intact or wounded cotyledons or hypocotyls on NAA/BA medium and rooted on IBA medium grew efficiently and produced new leaves after a few days of transfer to soil (78.3, 82.1, and 56.8% survival frequencies respectively). Significantly lower survival frequencies were observed for

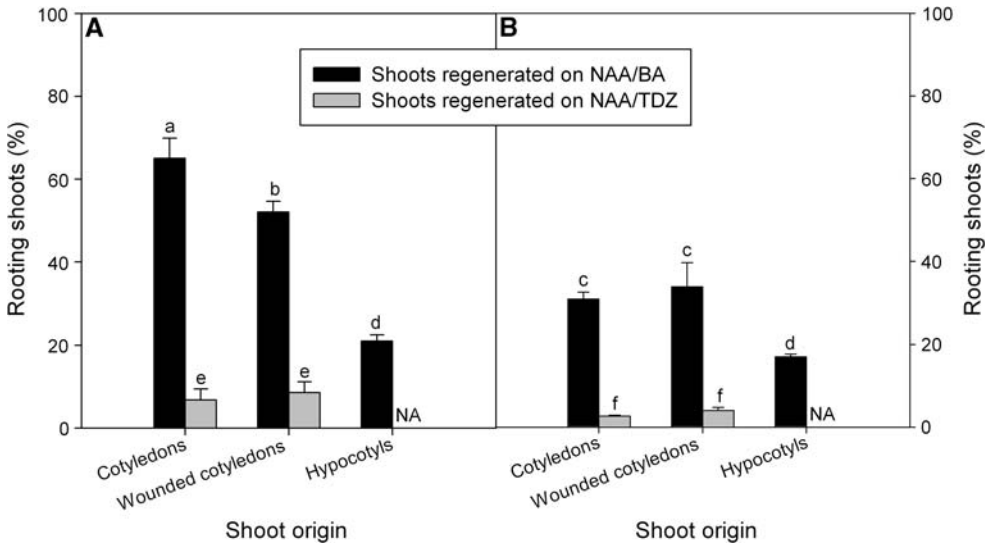

Fig. 5 Shoots regenerated on NAA/BA or NAA/TDZ media were rooted on IBA medium (**a**) or NAA medium (**b**). *Bars* are the average frequencies of root regeneration from six replicates (%) plus or minus SE. Counts were taken 3 weeks after subculturing onto the rooting medium. In both graphs, *bars* labeled with different *small letters* are significantly different at $P < 0.5$. NA on the *x*-axis of **a** and **B** means not applicable

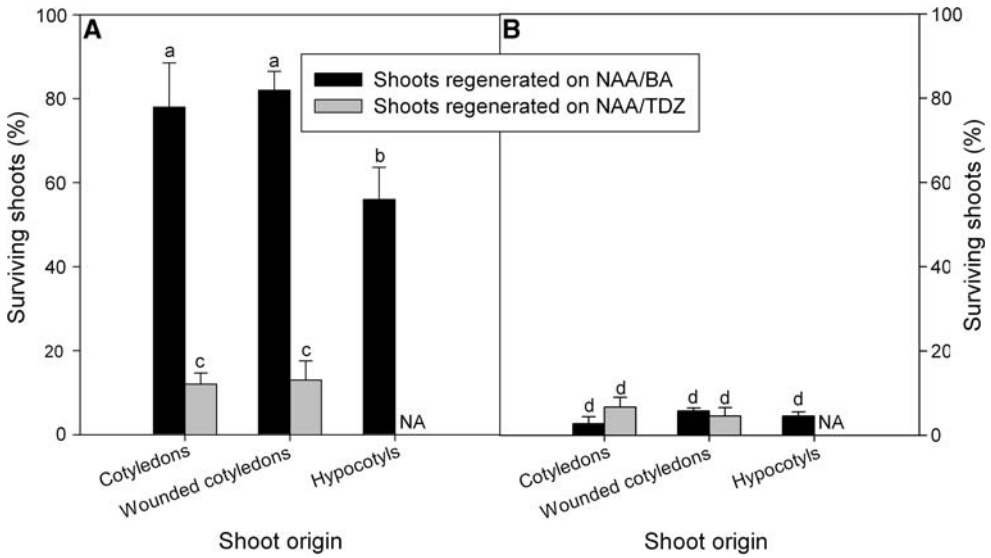

Fig. 6 survival of shoots regenerated on NAA/BA or NAA/TDZ media and rooted on IBA (**a**) or NAA (**b**) media. *Bars* are the average frequencies of survival (%) plus or minus SE. Counts were taken 10 days after plantlet transfer to soil. In both graphs, *bars* labeled with different *small letters* are significantly different at $P < 0.5$. NA on the *x*-axis of **a** and **b** means not applicable

shoots regenerated from intact or wounded cotyledons on NAA/TDZ medium and rooted on IBA medium (12.7 and 13.2% respectively). Very low frequencies of survival were obtained for shoots rooted on NAA medium regardless of the shoot origin or the type of cytokinin used for shoot regeneration (maximum of 6.0%). Moreover, no significant survival differences were observed between shoots that have been regenerated on NAA/BA or NAA/TDZ media and rooted on NAA medium.

Regeneration of Sakha3 and Sakha4

Shoots from the cultivars Sakha3 and Sakha4 were regenerated on NAA/BA medium containing 0.05 mg l^{-1} NAA and 2.0 mg l^{-1} BA that proved efficient for regeneration of the landrace Mescawi-ahaly. Sakha3 showed significantly higher shoot regeneration from intact and wounded cotyledons as well as from hypocotyls than Sakha4 (Table 1). The highest frequencies of shoot regeneration for Sakha3 and

Table 1 Regeneration of Sakha3 and Sakha4 shoots from different explants on NAA/BA media followed by rooting on IBA medium

Cultivar	Explant	Shoot regeneration frequency (%)	Root regeneration frequency (%)	Survival (%)
Sakha3	Intact cotyledon	71.45 ± 6.76 a	61.35 ± 4.61 a	71.51 ± 4.97 a
	Wounded cotyledon	11.43 ± 3.54 d	37.81 ± 2.08 c	68.65 ± 5.93 a
	Hypocotyl	27.54 ± 1.65 c	22.98 ± 4.87 d	21.65 ± 4.76 b
Sakha4	Intact cotyledon	45.65 ± 7.64 b	49.15 ± 6.76 b	66.54 ± 3.62 a
	Wounded cotyledon	4.55 ± 2.56 e	21.65 ± 2.65 d	63.76 ± 7.65 a
	Hypocotyl	6.64 ± 0.65 e	17.65 ± 2.03 d	27.56 ± 6.97 b

Values are shoot or root regeneration or survival frequencies plus or minus SE

Mean values ±SE within each column with different letters are significantly different at $P < 0.05$

Table 2 Time scale of whole plantlet regeneration of *Trifolium alexandrinum* L. from different types of explants on NAA/BA medium

Explant	Shoot initiation	Shoot development	Rooting	Total
Intact cotyledon	3.0	1.5	3.0	7.5
Wounded cotyledons	3.0	1.5	3.0	7.5
Hypocotyls	4.0	2.0	4.0	10.0

Values are the amount of time required for each step in weeks

Sakha4 were obtained from intact cotyledons (71.4 and 45.6% respectively). Wounded cotyledons and hypocotyls produced significantly lower regeneration frequencies in both cultivars. Rooting of the regenerated shoots was performed on IBA medium (0.24 mg l^{-1} IBA). In general, Sakha3 showed higher rooting frequency than Sakha4 (Table 1) and the highest rooting frequencies were observed for shoots regenerated from intact cotyledons (61.3 and 49.1% respectively). No significant differences were found in the survival frequencies of shoots regenerated from intact and wounded cotyledons from Sakha3 and Sakha4 (ranged from 63.7% in Sakha4 to 71.5% in Sakha3). However, the survival frequencies of shoots regenerated from hypocotyls were significantly lower in both cultivars than these regenerated from intact or wounded cotyledons (21.6% for Sakha3 and 27.5% for Sakha4).

Time scale of whole plant regeneration

The amount of time required for whole plant regeneration in the three tested genotypes depended on the type of explant with up to 7.5 weeks for intact and wounded cotyledons and 10 weeks for hypocotyls (Table 2).

Discussion

The genus *Trifolium* includes many important forage crops in addition to Egyptian clover. Protocols have been described for tissue culture regeneration and/or transformation of several *Trifolium* crops (Khan et al. 1994; Voisey et al. 1994; White and Voisey 1994; Beattie and Garrett 1995; Larkin et al. 1996; Quesenberry et al. 1996; Ding et al. 2003). However, information on the regeneration of Egyptian clover is very rare. To date, *A. tumefaciens*-mediated transformation of Egyptian clover has never been reported. This could be essentially due to the unavailability of a reliable time-efficient protocol for tissue culture regeneration of this crop.

In this study, we describe new methods for regeneration of Egyptian clover. Three genotypes of Egyptian clover (the landrace Mescawi-ahaly and the two cultivars Sakha3 and Sakha4) were successfully regenerated from intact and wounded cotyledons as well as hypocotyls. The regeneration procedure was optimized for Mescawi-ahaly (which is the most cultivated genotype in Egypt, unpublished data) and then the optimized conditions were used to regenerate Sakha3 and Sakha4. The use of intact cotyledon explants for shoot regeneration has never been described before for Egyptian clover although it has been described for several other species of *Trifolium* (White and Voisey 1994; Ding et al. 2003). In this study, intact cotyledons showed the highest shoot regeneration frequency for Mescawi-ahaly compared to wounded cotyledons or hypocotyls either on NAA/BA or NAA/TDZ media (Figs. 2, 3, 4). Such regeneration did not involve extensive formation of callus but rather involved slight swelling of the cotyledon stalk followed by appearance on its cut end of meristemoids that developed into shoots (Fig. 1a–e). Similar regeneration has been detailed for white clover by White and Voisey (1994). The origin of meristemoids might be some meristematic cells from the shoot meristem (at the hypocotyl node) of the seedling that were included in the explant. When the cotyledons were cut above the hypocotyl node, the regeneration was diminished (data not shown). Cotyledons older or younger than 3 days gave less regeneration frequencies, but the decrease was more severe in older cotyledons (data not shown). Half-cotyledon explants have been tested for shoot regeneration of four cultivars of Egyptian clover

including Sakha3 and Sakha4 in addition to Giza6 and Giza10 (Barakat 1990). The regeneration was successful only in Giza10 and it involved callus formation followed by shoot regeneration on different media. The poor shoot regeneration from half cotyledons could have been due to the small explant size (Kane 1990; George and Debergh 2008). For this reason, we used full but wounded cotyledons instead. The shoots regenerated directly from the cut edges on the cotyledon blade without intermediary callus formation (Fig. 1f–i) in contrast to the regeneration described by Barakat (1990) in which the shoots regenerated from intermediary calli. However, the shoot regeneration frequencies and the numbers of shoot per explant from the wounded cotyledons were lower than these from intact cotyledons on all regeneration media and for all genotypes (Figs. 2, 3; Table 1). Hypocotyls have been used for callus induction followed by shoot regeneration of Egyptian clover. However, the site of callus formation on the hypocotyl was not specified (Mokhtarzadeh and Constantin 1978; Barakat 1990). In our study, calli were also formed on the proximal cut ends of the hypocotyls but they did not contribute to the shoot regeneration, which occurred exclusively at the distal ends of the hypocotyls perhaps by multiplication of the original shoot meristems. When 5 days old hypocotyls where used, similar shoot regeneration frequencies were obtained. However, lower shoot regeneration frequencies were observed with hypocotyls younger than 4 days or older than 5 days (data not shown).

Two different media were used for shoot regeneration namely, NAA/BA and NAA/TDZ media. These media have been used for shoot regeneration of several species of *Trifolium* (excluding Egyptian clover; White and Voisey 1994; Beattie and Garrett 1995; Ding et al. 2003). In the present study, NAA/BA medium with very low concentration of NAA (0.05 mg l^{-1}) supported significantly higher shoot regeneration than NAA/TDZ medium from intact and wounded cotyledons (Figs. 2, 3). Furthermore, hypocotyls failed to regenerate at all on NAA/TDZ medium although they regenerated shoots at considerable frequencies on NAA/BA medium (Fig. 4). A possible reason for this could because TDZ mimics the effects of both auxin and cytokinin on shoot regeneration, thereby producing higher auxin effect which in turn reduces the frequency of shoot regeneration (Jha and Ghosha 2005). The type of shoot regeneration media also affected shoot elongation (data not shown), the rooting capacity of the shoots as well the survival frequency of the plantlets, where the shoots regenerated on NAA/BA media showed significantly higher shoot elongation and rooting and survival frequencies compared to these regenerated on NAA/TDZ media (Figs. 5, 6). This indicates that TDZ may able to induce shoot regeneration but the shoots may suffer from certain abnormalities such as hyperhydricity and retarded growth that could reduce their rooting capacity particularly at higher concentrations of TDZ (Malik and Saxena 1992; Tabrett and Hammatt 1992; Lu 1993, Ledbetter and Breece 2004; Basalma et al. 2008).

Root induction from shoots was performed on either on 2.0 mg l^{-1} NAA that has been tested for Egyptian clover (Barakat 1990) or 0.24 mg l^{-1} IBA that has been tested for other species of *Trifolium* (Beattie and Garrett 1995). The rooting frequency on IBA medium was significantly higher than that on NAA medium except for shoots developed from hypocotyls where it was similar on both media (Fig. 5). Furthermore, NAA medium induced callus formation at the shoot bases from which some roots were also induced. Such response was not observed on IBA medium. This resulted in (or at least contributed) to the significantly lower survival frequencies for shoots rooted on NAA medium compared to these rooted on IBA medium (Fig. 6). Although NAA rooting medium has been reported specifically for Egyptian clover, IBA rooting medium that has been reported for other types of clover proved more efficient for root induction in Egyptian clover.

Based on the previous observations, it is obvious that shoots of Egyptian clover are best regenerated from intact or wounded cotyledons on NAA/BA medium (0.05 mg l^{-1} NAA and 2.0 mg l^{-1} BA) for 3 weeks followed by 10 days on SD medium for further shoot development. Shoots could also be regenerated from hypocotyls on NAA/BA medium (2.0 mg l^{-1} NAA and 0.5 mg l^{-1} BA) for 4 weeks and then subcultured onto SD medium for 2 weeks. Rooting of the shoots could be induced on IBA medium (0.24 mg l^{-1}) for 3–4 weeks (according the origin of the shoot) followed by transfer of plantlets to soil. When these optimized conditions were applied to the cultivars Sakha3 and Sakha4, intact cotyledons showed the highest shoot and root regeneration frequencies in both cultivars although the regeneration frequencies were significantly higher in Sakha3 (Table 1). Wounded cotyledons showed very low shoot regeneration frequencies in both cultivars although the survival of the shoots was similar to these from intact cotyledons.

Intact cotyledons seem to be the best explant for regeneration of the three genotypes of Egyptian clover tested in this study with full plantlets regenerated after 7.5 weeks from culture initiation (Table 2). It was not possible to compare the efficiency of this regeneration procedure to the previously described ones (Mokhtarzadeh and Constantin 1978; Barakat 1990) because no regeneration frequency or time scale information was provided in those reports. However, the amount of time required for regeneration of Egyptian clover from *A. rhizogenes*-transformed material has been reported to be at least 4 months (Tanaka et al. 2001).

The present time-efficient regeneration procedure, utilizing cotyledons has never been described before for Egyptian clover. Moreover, cotyledons have been described as well suited targets for *Agrobacterium*-mediated transformation (Ding et al. 2003), and therefore the regeneration procedure described in this study should be useful for transformation of Egyptian clover.

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