Establishment of effective regeneration system in Egyptian-adapted canola var. pactol

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Canola (*Brassica napus* L.) is the oilseed crop of cold season countries, yet recently new varieties are being tested in warmer-climate countries. In Egypt, new varieties have been introduced / tested for adaptation to Egyptian environment, therefore gaining the interest of workers in the field of oil-crops production. In the current work, canola var. Pactol, a well adapted variety to Egyptian environment, was tested for regeneration ability *via* direct and indirect shoot organogenesis, as the first step for further crop improvement. Hypocotyls segment were tested on different media; best results for direct shoot organogenesis was achieved on medium fortified with 0.45 mg/l Thidiazuron + 3.0 mg/l benzyl adenine and 0.1 mg/l Naphthalene acetic acid (88.3%), with an average shoot number of 10.5 ± 1.23. For indirect shoot organogenesis, (and after an initial incubation of 10 days on 2, 4-dichlorophenoxy acetic acid fortified medium), the best results were obtained on medium supplemented with 0.15 mg/l Thidiazuron + 1.0 mg/l benzyl adenine and 0.5 mg/l naphthalene acetic acid (73.75%), with an average shoots of 7.38 ± 0.17. Achieving a reliable regeneration system is a step forward in production of genetically enhanced dehydration-stress tolerance varieties able to grow in new reclaimed area in Egypt.

Keywords: Canola regeneration; oil-crops; direct organogenesis; Thidiazuron.

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Introduction

Vegetable oils are, and have been an important component of human diet for long time. Nowadays, the four major vegetable-oil producing crops (oil-palm, soybean, rapeseed and sunflower, respectively) cover about 270 million hectares, with a production of 170 million tons, and accounting for approximately 75% of world total production [1]. Canola (*Brassica napus* L.) is an important member of *Brassicaceae* family; a family that includes several important genera. There are six *Brassica* species, three of those are diploid *B. rapa* (AA genome type, 2n= type, 2n=20), *B. nigra* (BB genome type, 2n=16), and *B. oleracea* (CC

genome type, 2n=18) which have hybridized to give rise to three amphidiploids {*B. napus* (AACC, 2n=38), *B. juncea* (AABB, 2n=36), and *B. carinata* (BBCC, 2n=34)} [2, 3]. The family *Brassicaceae* includes several individuals of economically important and growing through-out the world.

In general, Egypt's production of vegetable oils covers only a small portion of its current demand. According to Hassan and Shafique [4], Egypt's self-sufficiency of vegetable oil declined from 95 % in early 1960's, to less than 30 % in 2004, leading to increase importation of processed vegetable oil and semi-processed oil-seeds. In 2011, Egypt spent about 1.875 billion US dollars on importation of vegetable oils combined [5]. The United States Department of Agriculture, division of Foreign Agriculture Service (USDA-FAS), reported in 2013-2014 that Egypt's total oilseeds planted area only covers 3-5% of Egypt's total edible oil consumption. Expanding in edible-vegetable oil production in Egypt is limited due to limitation of agriculture land and water supplies. Out of about 3.7 million hectares available for food production, it is estimated that 3.2 million hectares is dedicated for cereal crops. Therefore, expanding cultivation into new reclaimed areas, while developing new high-yielding varieties, as well as, minimizing agricultural inputs might be the only formula for Egypt to meet its need of food.

One major problem that could hinder any increase in oil-crop production efforts would be the quality of irrigation water. In general, irrigation in the majority of new reclaimed area depends on underground water, which usually contains higher level of Na^+ ions compared to the Nile river water [6]. Salinity causes adverse damages on crops, especially that the majority of known crops are sensitive to high concentration of Na^+ ions [7]. Therefore, imbedding dehydration-stress tolerance traits, along with high-yield components in new varieties could be a major step toward minimizing the gap in vegetable oil production in Egypt.

Canola has been the target of interest for many researchers worldwide [8-26]. Canola is an amenable plant to tissue culture, yet regeneration success is greatly influenced by several factors, as genotype, explant type and media composition, that eventually affects the regeneration procedure.

Different genotypes were screened for their ability to produce healthy green shoots, yet percentage of success varied among different genotypes [13, 14, 17, 18, 20, 22], with a success rate that ranges from 0 up to 100%.

Different explant types were tested to achieve successful regeneration in *Brassica napus*, as in stem segments [22, 27, 28], thin leaves-cell layer [19, 29], leaf discs [16, 30], roots [18, 31], protoplast [11], cotyledons [9, 10, 18, 20, 25, 32-34], and hypocotyls [8, 12-15, 17, 18, 20-26, 35, 36, 37].

Interestingly, pretreatment of canola explants for different time period (ranged from few days up to 3 weeks), on media fortified with growth regulators was reported by different groups in order to promote calli formation [13-15, 17, 18, 21, 24, 38-40], 4-dichlorophenoxy-acetic acid (2,4-D) has been the most common growth regulator used for callus induction on its own [15, 17, 38-40], or when combined with other growth regulators [18, 21, 24]. On the other hand, few reports suggested that successful callus induction could be achieved without the need of explants pretreatments [20, 22].

As a first step in developing genetically–enhanced varieties *via* plant transformation, and as a first step, we are reporting the establishment of a reliable, direct regeneration system in canola cv. Pactol, by investigated two scenarios for plant regeneration, indirect shoot formation "the most common in literature" and direct shoot formation.

Materials and Methods

Plant material and regeneration

Canola seeds (Brassica napus L. var. Pactol), a variety that is well-adapted to Egyptian environment, were obtained from Field Crops Research Institute (FCRI), Agriculture Research Center (ARC), Giza, Egypt. All fine chemicals and media used in this study, unless otherwise stated, were purchased from (Duchefa, Haarlem, Netherlands).

Regeneration

Mature seeds of canola var. "Pactol" were surface sterilized by soaking in 70% (v/v) ethanol for 1 min, followed by soaking for another 15 min in 20% commercial Clorox (1.0 % sodium hypochlorite solution) with a drop of Tween® 20 with stirring. Seeds were washed 5-6 times with sterilized distilled water to ensure the removal of disinfecting residues. Seeds were germinated for 5 - 6 days on 1/2 strength MS medium [41], supplemented with Gamborg's B5 vitamins [42] + 10.0 g/l sucrose and 7.0 g/l agar. The pH of this medium and all subsequent media were adjusted to 5.7, followed by autoclaving at 121˚C for 20 min. All tissue culture materials unless otherwise mentioned were kept in growth chamber at 25 ± 2˚C for 16/8hr light/dark photoperiod.

Hypocotyls of 0.5 - 1.0 cm long were excised using scalpel then cultured on shoot initiation media. Twenty different shoot initiation MS-based media were tested. All media consisted of MS basal salt mixture supplemented with Gamborg's B5 vitamins with 15.0 g/l sucrose and 6.5 g/l agar, as shown in Table 1. Two different approaches were used:

(1) Direct regeneration: where hypocotyls were placed on the medium directly for regeneration. This experiment included 20 treatments. The media consisted of MS basal salt mixture supplemented with different concentrations of Thidiazuron (TDZ), Benzyl adenine (BA), and Naphthalene acetic acid (NAA) (Table 1). All experiments, unless otherwise stated, had 4 replicates in each treatment, and 10 explants per replica. The experiment was repeated three times. Shoots were counted after 4-5 weeks before sub-culturing onto fresh medium.

(2) Indirect regeneration: excised-hypocotyls were placed on medium consisting of MS basal salt mixture with Gamborg's B5 vitamins + 1.0 mg/l 2, 4-dichlorophenoxy-acetic acid (2, 4-D) + 30.0 g/l sucrose and 7.0 g/l agar for 10 days before being transferred onto the shoot regeneration media. Shoots were counted after 2-3 weeks.

Well-developed shoots were carefully excised with scalpel and transferred onto hormone-free MS basal salt mixture medium supplemented with Gamborg's B5 vitamins + 15.0 g/l sucrose and 6.5 g/l agar; 1-2 weeks later, healthy shoots were excised again "to remove explant residues and unwanted tissues" and were placed onto rooting medium consisting of 1/2 strength MS medium supplemented with 0.1 mg/l Indol butyric acid (IBA) + 5.0 g/l sucrose and 6.5 g/l agar. Healthy plantlets, with strong rooting system were transferred into pots containing a mixture of (clay : sand : peat moss) (1 : 1 : 1) in a Conviron® growth chamber optimized at 25 ± 2˚C and 16/8 hr light/dark photoperiod. At first plantlets were covered with plastic bags for a week then bags were gradually removed. Plantlets were watered every 2 days and fertilization was conducted with 5.0 g/l Kristalon ™ (N : P : K) at ratio of (19 : 19 : 19). After 2 months, plants were transferred to the green house, fertilized and irrigated until reaching maturity and seeds were obtained.

Results and Discussion

Direct shoot regeneration

Ten days after placing explants on the different treatments (Table 1), explants turned into dark green color and were at least 2 - 3 times bigger than the original starting materials, and the first

Table 1. Growth regulators combinations of the twenty MS- based shoot regeneration media used for canola var. Pactol shoot formation.

No	Media Name	Growth regulators (mg/l)	No	Media Name	Growth regulators (mg/l)
1	**MS1**	0.00 TDZ, 0.0 BA, 0.0 NAA	**11**	**MS11**	0.00 TDZ, 0.0 BA, 0.5 NAA
2	**MS2**	0.15 TDZ, 1.0 BA, 0.0 NAA	**12**	**MS12**	0.15 TDZ, 1.0 BA, 0.5 NAA
3	**MS3**	0.30 TDZ, 2.0 BA, 0.0 NAA	**13**	**MS13**	0.30 TDZ, 2.0 BA, 0.5 NAA
4	**MS4**	0.45 TDZ, 3.0 BA, 0.0 NAA	**14**	**MS14**	0.45 TDZ, 3.0 BA, 0.5 NAA
5	**MS5**	0.60 TDZ, 4.0 BA, 0.0 NAA	**15**	**MS15**	0.60 TDZ, 4.0 BA, 0.5 NAA
6	**MS6**	0.00 TDZ, 0.0 BA, 0.1 NAA	**16**	**MS16**	0.00 TDZ, 0.0 BA, 1.0 NAA
7	**MS7**	0.15 TDZ, 1.0 BA, 0.1 NAA	**17**	**MS17**	0.15 TDZ, 1.0 BA, 1.0 NAA
8	**MS8**	0.30 TDZ, 2.0 BA, 0.1 NAA	**18**	**MS18**	0.30 TDZ, 2.0 BA, 1.0 NAA
9	**MS9**	0.45 TDZ, 3.0 BA, 0.1 NAA	**19**	**MS19**	0.45 TDZ, 3.0 BA, 1.0 NAA
10	**MS10**	0.60 TDZ, 4.0 BA, 0.1 NAA	**20**	**MS20**	0.60 TDZ, 4.0 BA, 1.0 NAA

The experiment was repeated 3 times and each treatment consisted of 4 replicates.

Table 2. Regeneration of canola via direct and indirect shoots production; numbers with the same latter are insignificants.

	Direct shoot regeneration		**Indirect shoot regeneration**	
Treatment	**Shoot No.**	**Percentage**	**Shoot No.**	**Percentage**
MS 1	0.17 ± 0.10 j	1.60	0.25 ± 0.12 i	2.50
MS 2	2.00 ± 0.38 h	20.9	2.13 ± 0.50 e f	17.14
MS 3	2.67 ± 0.17 g	26. 7	2.38 ± 0.01 e	23.00
MS 4	5.98 ± 0.51 de	58.3	4.88 ± 0.18 c d	48.75
MS 5	5.50 ± 0.22 de	55.0	5.36 ± 0.30 c	53.75
MS 6	0.75 ± 0.08 i	7.50	0.63 ± 0.10 h	6.25
MS 7	8.70 ± 0.23 b	69.0	5.80 ± 0.56 c	54.20
MS 8	4.70 ± 0.11 de	41.8	3.54 ± 0.50 d	37.10
MS 9	**10.50 ± 1.23 a**	**88.3**	5.75 ± 0.60 c	57.50
MS 10	7.33 ± 0.49 c	66.7	6.60 ± 0.12 b	66.25
MS 11	0.08 ± 0.05 j	1.00	0.00 ± 0.00 j	00.00
MS 12	6.83 ± 0.37 cd	68.3	**7.38 ± 0.17a**	**73.75**
MS 13	5.33 ± 0.46 de	46.7	4.38 ± 1.10 cd	43.75
MS 14	5.00 ± 0.76 def	50.0	5.25 ± 0.60 c	37.50
MS 15	4.00 ± 0.76 ef	51.0	5.25 ± 0.70 c	52.50
MS 16	0.08 ± 0.05 j	1.0	0.13 ± 0.10 i	1.25
MS 17	1.36 ± 0.46 hi	15.0	1.50 ± 0.08 f	15.00
MS 18	0.89 ± 0.32 i	10.0	1.54 ± 0.57 f	17.00
MS 19	1.58 ± 0.24 h	15.8	0.87 ± 0.06 g	8.75
MS 20	1.71 ± 0.33 h	17.1	1.75 ± 0.47 f	17.50
Mean	3.76		3.27	

Numbers in this table represents mean ± SE; values followed with similar letters are non significant at $p \leq 0.05$.

set of shoots and shoot primordial were observed after 14 days (Figure 1). Interestingly, shoots primordia did not only appear from cutting edges of explants, but also from the sub-epidermal tissues of hypocotyls explants, similar to previous reports [14, 20, 22]. In general, regeneration frequencies ranged from 1.0 up to 88.3 % (Table 2 and Figure 1), with treatment 9 being the best treatment (with the highest average in shoot number/explant (10.5 ± 1.23, Table 2). This was followed by treatments 7 and 10 (8.7 ± 0.23 and 7.33 ± 0.49, respectively).

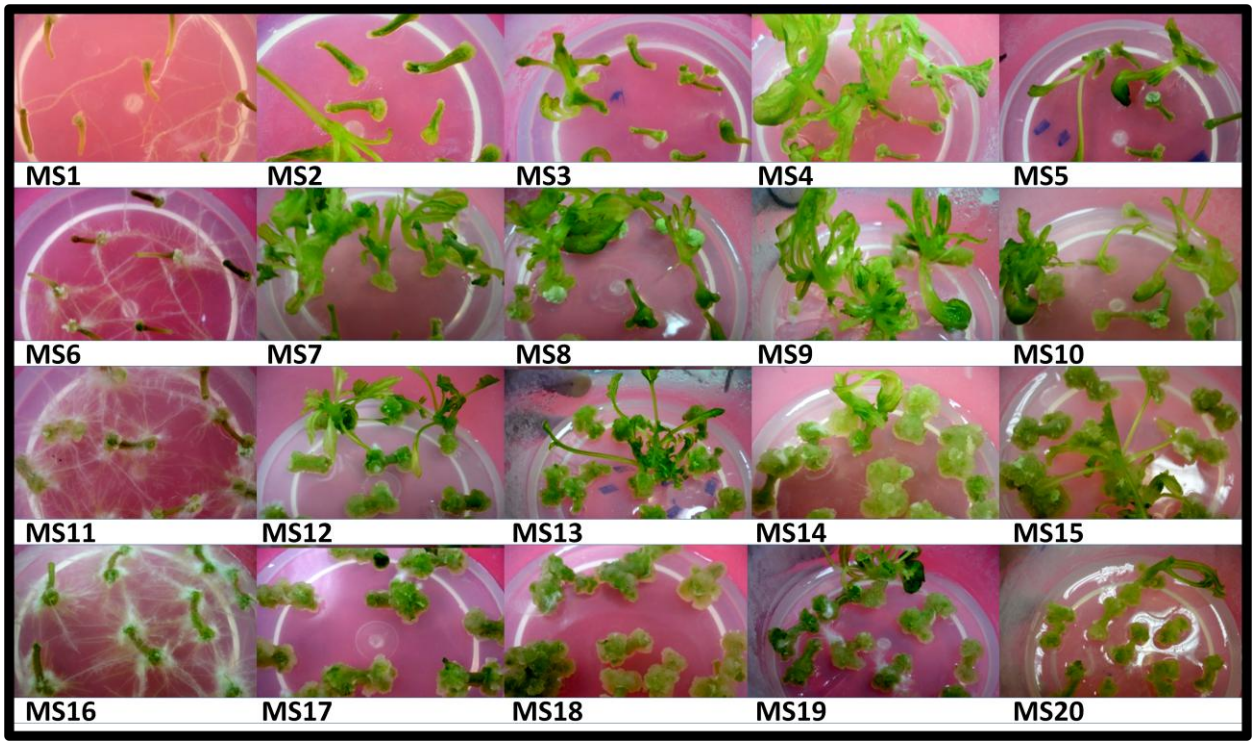

Figure 1. Regeneration in canola var. Pactol via direct-shoot organogenesis; pictures were taken 4 weeks post incubation on different regeneration media and represents a sample of explant response to treatments.

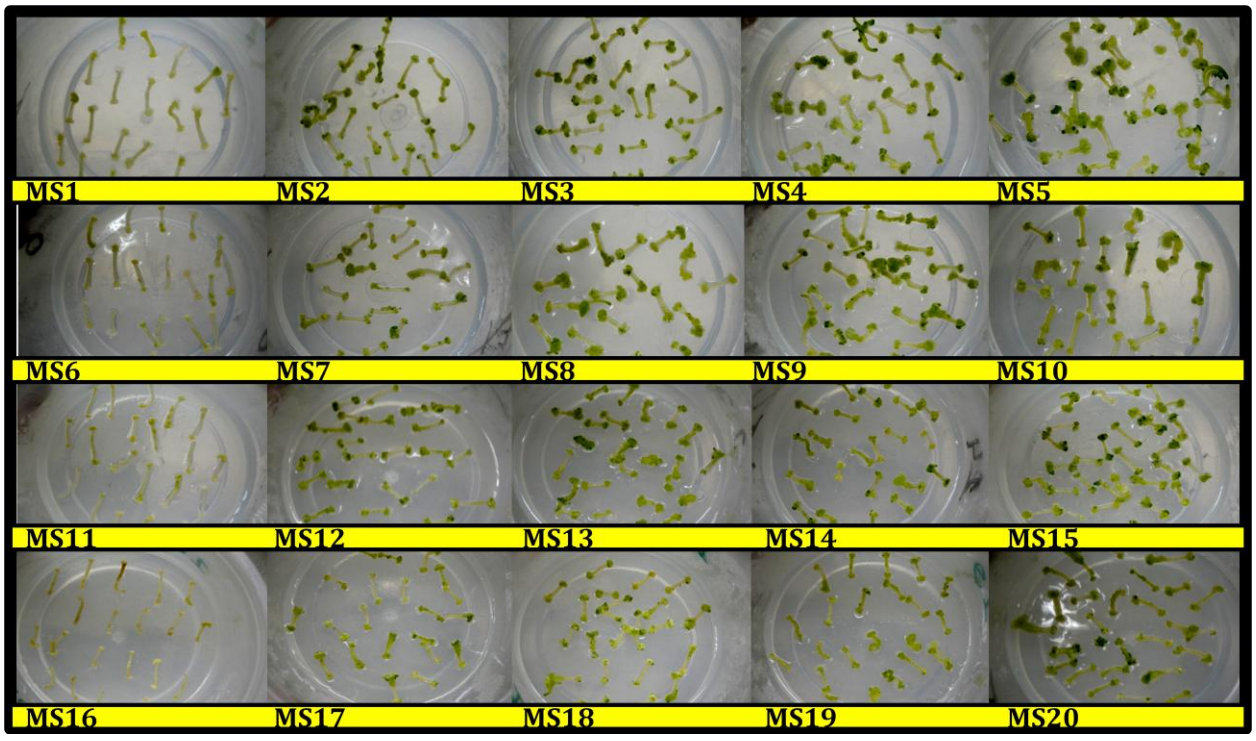

Figure 2. Regeneration in canola var. Pactol via indirect shoot formation; pictures represents a sample of explant responding to different media treatments almost 3 weeks post starting of regeneration process (10 days incubation on 2,4-D containing medium +11 days on the different regeneration media).

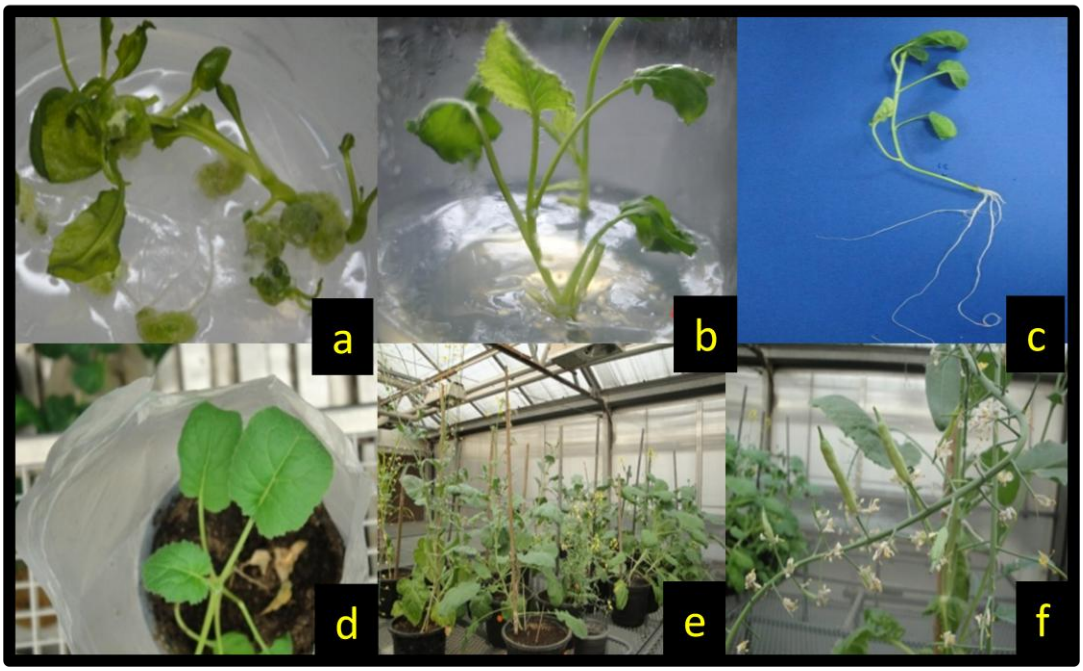

Figure 3. Different developmental stages of canola shoots. (a) Vitrified canola shoots, (b) Shoots recovering and elongated on hormone-free medium, (c) Plantlets of canola after root formation on 1/2 strength MS medium supplemented with IBA, (d) Acclimatization in growth chamber, (e) Plants in greenhouse, (f) Seed-setting in greenhouse.

In general, regeneration in canola via direct shoot regeneration was achievable when using cytokinins (BA and TDZ) in the media (with an average mean of 3.26 vs. 0.27 shoots, in the absence of cytokinins). Yet upon the addition of NAA to the regeneration media (in low concentration, 0.1 mg/l), almost a double increase in regeneration frequency was achieved (an average mean of 6.39 vs. 3.26 without NAA). The same trend of results has been previously reported in canola upon the addition of low concentrations to the media [15, 17, 20, 22, 23, 24, 34).

Indirect shoot regeneration

Indirect shoot formation in canola was achieved by placing the hypocotyl explants on medium containing 2, 4-D, at a concentration of 1.0 mg/l for 10 days, and was immediately placed on different medium (Table 1). The incubation period on 2, 4-D medium didn't cause enlargement in the initial explant size and explants were whitish in color in most treatments (Figure 2). Transferring the explants into the different regeneration medium resulted in green color formation in explants and in later stages, darker green spots were formed. Maximum regeneration was achieved on MS12 medium, with an efficiency of 73.75% (Table 2). A significant decrease was observed in regeneration frequency upon the increase of NAA concentration from 0.1 to 1.0 mg/l, which is in agreement with the results of Khan et al. [13, 14] and Tarinejad [24], while in contrast to results obtained by Kamal et al. [18].

Interestingly, most of the literatures on canola regeneration focused on indirect shoot formation via organogenesis as being the most successful in achieving regeneration [13-15, 17, 18, 21, 34, 38-40]. Some researchers reported that 2, 4-D was the most effective in producing callus for a period from 7-20 days [13-15, 17, 38-40, 43]. Other researchers identified that incubation on 2, 4-D alone was not sufficient enough, therefore decided to combine 2, 4-D with other growth regulators such as NAA and Kinetin [18]; other workers decided to omit 2, 4-

D from their media and used different combinations of growth regulators [21, 24, 34].

In general, canola shoots coming from direct and indirect regeneration showed some degree of vitrification (Figure 3a), indicating an imbalance of water content in tissues. Therefore, shoots were transferred to a hormone-free MS medium for 1-2 weeks to recover. Upon shoot recovery, and formation of new healthy leaves (figure 3b), shoots were transferred to a fresh 1/2 strength MS medium supplemented with 0.1 mg/l IBA + 5.0 g/l sucrose and 6.5 g/l agar, where they started forming roots (Figure 3c). Our results are also in line with previous reports indicating the extensive usage of IBA in canola root-formation [18, 23, 24, 34, 38, 40].

We also observed that placing canola shoots on full strength MS medium with IBA resulted in reduction in root formation, compared to using 1/2 MS medium (data not shown) which indicate that lower salt concentration in the medium favors root formation; also decreasing sucrose concentration from 30.0 to 5.0 g/l had positive effect on inducing root formation (data not shown).

Successfully rooted-shoots (with strong rooting system) were trans-planted into small pots containing moisture soil, covered with plastic bags and were acclimatized in Conviron® units (figure 3 d). Plant hardening was carried out by partial removal of plastic bags, and periodic exposure of the plants to natural environment. Plants were then transferred to greenhouse, where they grew normally and were able to flower and set seeds (Figure 3e, f).

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