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Optimization of Callogenesis from Khardel Aswed *Brassica nigra* L. local Iraqi cultivar

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Abstract

The current study was conducted to optimize callogenesis protocol for local cultivar of Brassica nigra. The results showed that all the sterilization methods were effective in controlling seed contamination. However, there were noticeable effects for the sterilization methods on the viability of the seeds. The treatment 70% ethanol for 1 minute followed by 30% Fas (contain 6% sodium hypochlorite) for 10 minutes was the best for seeds sterilization. Callus was induced from different explants from In vitro growing seedlings and they respond differently on the different callus induction media. The true leaf and the root explants gave hairy roots on MS medium supplemented with 0.5 mg.L^{-1} NAA + 2 mg.L^{-1} Kin which reduced the percentage of callus induction to 4% for both explants. The results also indicated that stem followed by cotyledon showed high percentage of callus induction than other explants (35%, 31%) respectively. Among the three callus induction media, MS medium supplemented with 2 mg.L^{-1} 2, 4-D and 2 mg.L^{-1} Kin was the most responsive medium with an average of 40.25% callus induction. The interaction effect of the media and the explants on callus fresh and dry weights showed that stem explants grown on MS medium supplemented with 2 mg.L^{-1} 2, 4-D and 2 mg.L^{-1} Kin gave significantly higher callus fresh and dry weights than other combinations and followed by cotyledon. According to callus color the calli that were induced from different explants on different media under light condition varied in color such as green, brown, pale yellow and greenish white. In conclusion stem and cotyledon of Khardel are the best explants for callus induction on MS basal medium supplemented with 2 mg.L^{-1} 2, 4-D and 2 mg.L^{-1} Kin.

Keywords: *2, 4-D, Kin, NAA, stem, cotyledon, hairy roots, callus induction*

Introduction

Brassica nigra L. (The common names are "Mustard" in English,"Sarson" in Hindi and Khardel Aswad in Arabic) is an annual weedy medicinal plant belongs to the family (Brassicaceae) and adapted in many environments. However, it has a very limited cultivation in the northern districts of Iraq (H.L. Chakravarty, 1976).

Tissue culture was adopted by many researchers for the propagation of many medicinal plants to overcome environmental and seasonal problems. The type and concentration of plant growth regulators and the explants types have great effect on callus induction (P. Pandey *et al*., 2013). S. Govil *et al*. (1986) developed calli from anthers of *Brassica nigra* which were cultured on B_5 medium containing different concentrations of BAP (benzylaminopurine) and 2, 4-D (2, 4-dichlorophenoxyacetic acid). Embryogenic callus of *Brassica nigra* was also obtained from hypocotyl explants on MS medium containing (kin) and (2, 4-D) (V. Gupta *et al*., 1990). In addition, U. J. Mehta et al. (1993) reported that callus of *Brassica nigra* is initiated from hypocotyls and roots explants and regeneration occurred via somatic embryogenesis. Another study reported callus induction with hairy roots from cotyledons, nodes and roots of *Brassica nigra* while hypocotyls gave callus only in medium supplemented with 0.5mg/l NAA and 2.0 mg/l Kin (J. Das *et al.*, 2010). Moreover, E. A. Hussein *et al.* (2010) obtained callus from hypocotyl explants of *Brassica nigra* on MS medium supplemented with a combination of (BA) and a mixture of (NAA) and (2,4-D). The vast researches indicated that callogenesis is affected by growth regulators, explants and the genotype. Therefore the objective of the current study was to optimize callogenesis conditions for local cultivar of *Brassica nigra*.

Materials and Methods

The present study was conducted at Genetic Engineering Department /Directorate of Agricultural Research of The Ministry of Science and Technology during the years 2014-2015. Khardel seeds of local cultivar were surface sterilized by submerging into 70% ethanol for 1 min;

Table 1: Composition of callus induction media used for *Brassica nigra*

Media type	Modified MS-Media Composition
MS1*	(4.91g/l) MS Salts + (30g/l) sucrose + (7g/l) Agar+ 2.5 mg/l (BA) + 0.5 mg/l (2,4-D) + 0.5 mg/l (NAA)
MS2	(4.91g/l) MS Salts + (30g/l) sucrose + (7g/l) Agar+ 2mg/l (2,4-D) + 2mg/l (Kin)
MS3**	(4.91g/l) MS Salts + (30g/l) sucrose + (7g/l) Agar+ 0.5 mg/l (NAA) + 2mg/l (Kin)
*E.A. Hussein *et al*., 2010 ** J. Das *et al*., 2010	

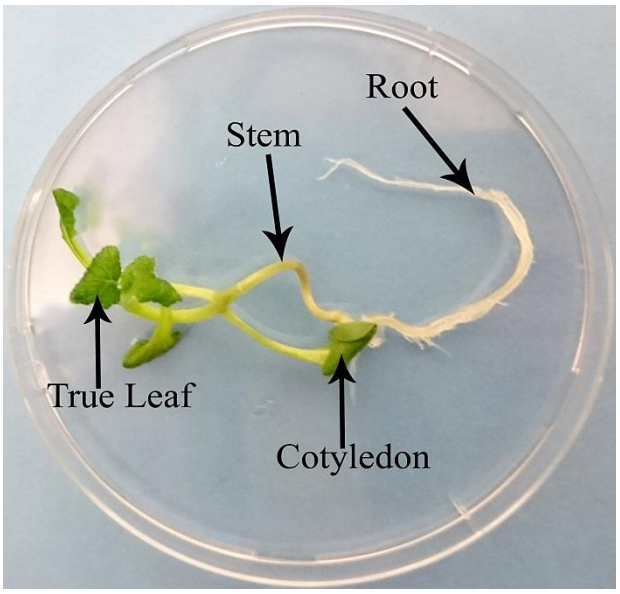

Figure 1 Seedling of *Brassica nigra*

washed with sterile distilled water for 5 min. Seeds were then sterilized for 5 or 10 min with (10%, 30% or 50%) of local disinfectant (Fas) which contains 6% sodium hypochlorite. Seeds were rinsed with sterile distilled water three times for 5 mins each. Effect of sterilization method on contamination and seed viability was tested by culturing sterilized seeds under aseptic conditions in plastic Petri dishes on hormone free readymade medium based on T. Murashige and F. Skoog (1962). Ten seeds were cultured in each Petri dish and 5 replications per treatment. The Petri dishes were incubated in the growth room at 21 ± 2ºC with 16/8 hours (light/dark) photoperiod of a light intensity 1000 lux provided by cool white fluorescence lamps. After 14 days of incubation seeds germination percentage (%) was calculated.

Callus was initiated from *In vitro* grown seedlings. Fully expanded leaves, cotyledon leaves, stems and roots were excised and cultured on basal MS media containing different combinations of growth regulators as shown in (Table 1, Figure 1). Five explants were cultured in plastic Petri-dishes with 10 replications per treatment. Petri dishes were incubated in the growth room conditions as above. After 4 weeks callus induction percentage (C_{ip}) was calculated using the following formula (M. A. Khater *et al*., 2013):

C_{ip} = number of explants forming callus/ total number of used explants x 100

After one month, callus fresh weight was measured and calli were dried in the oven at 60ºC until constant weight was obtained and it was recorded as a callus dry weight. The dry matter content was estimated according the following equation (M. A. Khater *et al.,* 2013):

Callus dry matter (%) = callus dry weight × 100/callus fresh weight

All the experiments were conducted in C.R.D (Completely Randomized Design) and the results were analyzed by GenStat software program (A. Glaser and C. Biggs, 2010). Means were compared by LSD at (0.05).

Results and Discussion

Sterilization of Seeds

The results showed that all the sterilization methods were effective in controlling seed contamination. However, there were noticeable effects for the sterilization methods on viability of the seeds (Table 2). The interaction analysis between the percentage of local disinfection solution (Fas) and sterilization time showed that the lowest percentage of seed germination was (26.00%) in 5 minutes exposure to sterilizing solution of 30%, whereas the highest percentage of seed germination was (86.00%) in 5 minutes exposure to 50% of sterilizing solution (Fig. 2). Fas solution contains 6.2% sodium hypochlorite which is the active disinfecting agent that has been widely used for surface sterilization in plant cell and tissue culture experiments (M. Yildiz and E. Celal,

2002; P. S. Warakagoda and S. Subasinghe, 2009; C. Derso and T. Feyissa, 2015). High concentration of sodium hypochlorite stimulate *Brassica nigra* seeds germination that could be due to the scarification effect on the seed coat that allows to improve the permeability for water and oxygen or due to the improvement of oxidative respiration by producing more oxygen through the decomposition of sodium hypochlorite (M. Yildiz and E. Celal, 2002).

Moreover, the results showed there were no significant differences between the treatment (50% Fas for 5 minutes) and 50% or 30% Fas for 10 minutes treatments in the percentage of seed germination (Table 2). Therefore the treatment 30% Fas for 10 minutes was used for sterilizing the seeds throughout this study.

Longer socking time in the disinfection solution improved water imbibitions of the seeds which improved the germination percentage.

Callus induction

The results showed that the explants respond differently on the different callus induction media (Table 3, Figure 3, 4). The true leaf and the root explants gave hairy roots on MS_3 media which reduced the percentage of callus induction to 4% for both treatments (Figure 3). Among the three callus induction media, MS_2 (MS medium supplemented with 2 $mg.L^{-1}$ 2, 4-D and 2 $mg.L^{-1}$Kin) was the most responsive medium with an average of 40.25% callus induction followed by the MS_1 with an average of 35.00%. However, both are significantly higher than MS_3 medium.

The results also indicated that stem, cotyledon and root explants showed significantly higher percentage callus induction than true leave explants (35, 31, 29.33%) respectively.

The interaction analysis between the media and the explants showed that there were no significant differences among root and true leaf explants grown on MS_2 medium and stem and cotyledon grown on MS_1 medium (Figure 4). Moreover these combinations were significantly higher than the other combinations in the percentage of callus induction (Table 3).

Table 2: Effect of Fas solution and sterilization time on the percentage of *Brassica nigra* seeds germination after 14 days

Sterilizing of solution % (Fas*)	Sterilization Time (minutes)		Mean
	5	10	
10	66.00	50.00	58.0
30	26.00	73.00	49.0
50	86.00	76.00	81.0
Mean	59.3	66.3	
L.S.D $_{(0.05)}$	Treat= 9.90 ; Time= 8.08 ; Interaction=14.00		

*Fas solution contains 6% sodium hypochlorite, *Each value in the table is the average of five replicates

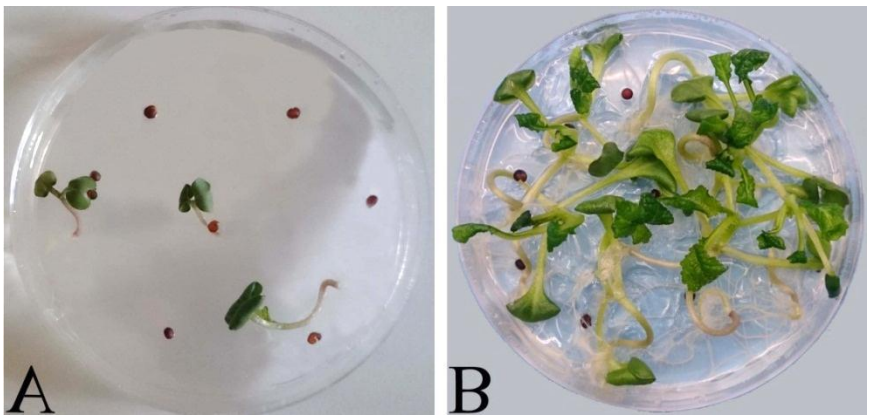

Figure 2 Effect of sterilization methods on seed germination: (A): 30% Fas for 5 minutes, (B): 50% Fas for 5 minutes

Table 3: The effect of media and explants on callus induction (percentage) of *Brassica nigra*

Media type	Explants				Mean
	True leaf	Cotyledon	Stem	Root	
MS_1	18.00	42.00	43.00	37.00	35.00
MS_2	44.00	32.00	38.00	47.00	40.25
MS_3	4.00	19.00	24.00	4.00	12.75
Mean	22.00	31.00	35.00	29.33	
L.S.D $_{(0.05)}$	Media= 6.81 ; Explants= 7.87 Interaction=13.63				

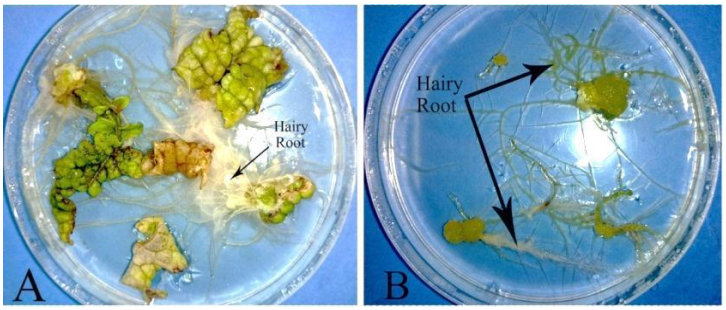

Figure 3 Formation of hairy roots from (A): True leaves, (B): Roots of *Brassica nigra* on (MS_3) MS supplemented with (0.5 $mg.L^{-1}$ NAA + 2$mg.L^{-1}$ Kin) after four weeks

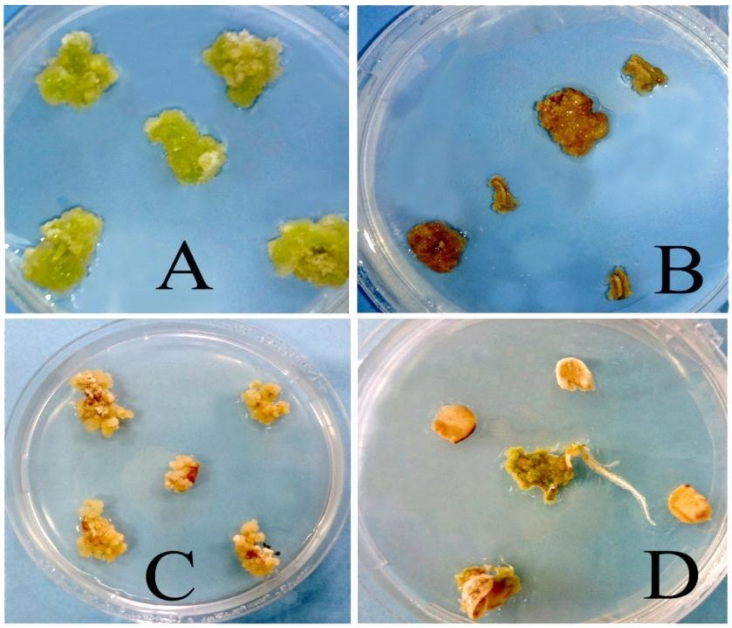

Figure 4 Callus induction of *Brassica nigra* from (A): Stem grown on MS_1 medium, (B): Stem grown on MS_3 medium, (C) Cotyledon grown on MS_1, (D) Cotyledon grown on MS_3 after four weeks

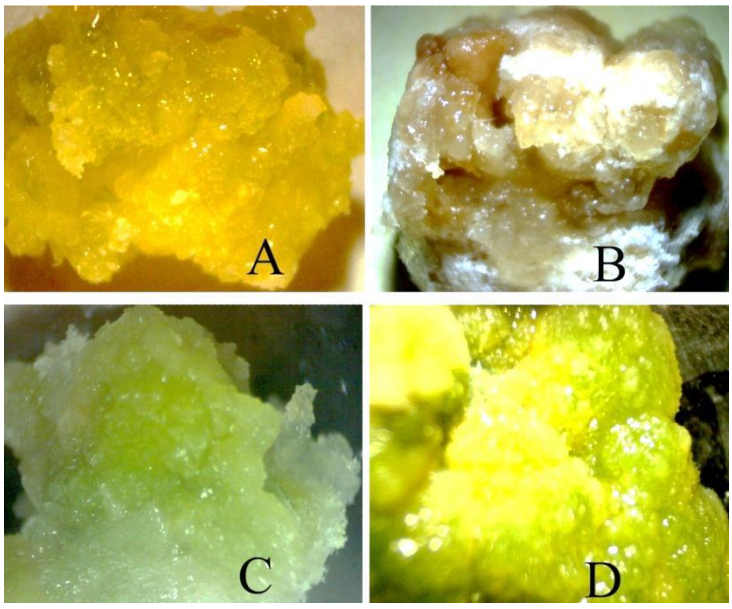

Figure 5 Callus varied in color induced from: (A) Root on MS_2 medium, (B) True leaf on MS_2 medium, (C) stem on MS_1 medium, (D) Cotyledon on MS_1 medium after four weeks

In the present experiment medium supplemented with NAA and Kin gave the highest percentage of hairy roots from true leaf and root explants. These findings are in agreement with J. Das *et al.* (2010) results' who obtained high frequency of hairy roots from root explants of *Brassica nigra* and maximum callus induction from hypocotyls on the same medium. Moreover, the present results was in agreement with E. A. Hussein *et al.* (2010) who reported that hypocotyl was the best explants for callus induction in *Brassica nigra* when incubated in medium containing 2.5 mg.L^{-1} BA , 0.5 mg.L^{-1} NAA and 0.5 mg.L^{-1} 2, 4-D combination. The results showed that equal amount of (kin) and (2, 4-D) gave high percentage of callus induction from root and true leaf, in contrast V. Gupta *et al*. (1990) reported embryogenic callus induction from hypocotyls explants of *Brassica nigra* when incubated on MS medium containing (kin) and (2, 4-D).

According to callus color (Figure 5) the calli that were induced from different explants on different media under light condition varied in color such as green, brown, pale yellow and greenish white. These colors were found in calli of all the explants grown on all the media combinations. Callus color depends on the pigments production of the cells under tissue culture conditions (W. S. Al- Manasrah, 2012). It has been found that growth regulators in the medium and the plant species affect the production of plant pigments in the callus cells which reflect the callus color appearance (H. Elias *et al*., 2014).

Effect of media and explants on the mean of callus fresh weights

The results showed (Table 4) that MS_1 medium produced the highest amount of callus fresh weight (557.5) mg which was significantly higher than the other media. Moreover, there were no significant differences ($p > 0.05$) between MS_2 and MS_3 in callus fresh weight with an average of (193.7, 219.0) mg respectively.

In terms of explants effect, stem explants gave the maximum amount of callus fresh weight with an average of (412.7) mg which was significantly different than the other explants. Whereas the cotyledon and root explants gave callus fresh weight with an average of (265.8, 291.6) mg respectively. True leaf explants were excluded from this analysis because most of them produced hairy roots with few callus amounts forming on the cut edge of the leaf.

The interaction analysis between the media and the explants showed that stem explants grown on MS_1 gave the highest callus fresh weight and the lowest was for cotyledon explants grown on MS_2 (Table 4).

Table 4 The effect of media and explants on the mean fresh weight (mg) of *Brassica nigra* callus after four weeks

Media type	Explants			Mean
	Cotyledon	Stem	Root	
MS_1	491.6	658.0	522.8	557.5
MS_2	102.8	363.6	114.6	193.7
MS_3	203.0	216.6	237.4	219.0
Mean	265.8	412.7	291.6	
L.S.D $_{(0.05)}$	Media= 141.3 Explants= 141.3 Interaction= 244.7			

The results of the current experiment indicated that callus induction and propagation from *Brassica nigra* are affected by the growth regulators combination and explants as with other plants. E. Sheeba *et al*. (2013) found that the highest callus induction from *Physalis minima* leaf explants was obtained on MS medium supplemented with NAA while the highest callus fresh weight was obtained from leaf explants in the presence of IAA and BAP. Furthermore, M. A. Khater *et al*. (2013) found that *Atropa belladonna* produced highly significant callus fresh weight from leaf explants in the presence of 2, 4-D only. While E. A. J. El -Kaaby (2015) reported that IAA and Kinetin had significant effect on callus mean fresh weight of chili pepper from shoot tips, cotyledonal leaves, hypocotyls and roots.

Effect of media and explants on the mean of callus dry weights

The results showed (Table 5) that the MS_1 medium was significantly higher in callus dry weight (52.80) mg compared with other media. In addition, there were no significant differences between MS_2 and MS_3 with an average of (23.00, 22.8) mg respectively.

The results (Table 5) also indicated that there were no significant differences between stem and cotyledon explants with an average of (39.80, 30.07) mg respectively. However, there were significant differences between stem and root explants with an average of (39.80, 28.80) mg respectively.

The interaction analysis between the media and the explants showed that stem explants grown on MS_1 gave the highest callus dry weight and the lowest was for root explants grown on MS_2 (Table 5).

The results of the current study were in disagreement with S. H. Tan *et al*. (2010) who reported that the combination of 2,4-D and kinetin in MS medium gave the highest callus dry weight. However, M. A. Khater *et al*. (2013) found that *Atropa belladonna* explants grown on media supplemented with 2,4-D alone gave maximum callus dry weight from leaf explants. On the other hand, S.

Table 5 The effect of media type and explants on the mean dry weight (mg) of *Brassica nigra* callus after four weeks

Media type	Explants			Mean
	Cotyledon	Stem	Root	
MS_1	51.20	57.80	49.40	52.80
MS_2	18.00	**36.20**	14.40	23.00
MS_3	21.00	25.40	22.60	22.8
Mean	30.07	**39.80**	28.80	
L.S.D $_{(0.05)}$	Media=10.04 Explants= 10.04 Interaction: 17.38			

Alagumanian *et al*. (2014) indicated that *Tylophora indica* produced maximum amount of dry weight from the nodal explants at 5 $mg.L^{-1}$ 2, 4-D alone and the minimum amount was obtained at 0 $mg.L^{-1}$.

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