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Development of an Efficient Regeneration Protocol for *Brassica carinata* A. Braun through *In vitro*-derived Explants

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Key words: Brassica carinata, *In vitro*, Regeneration

Abstract

Brassica crops are globally recognized for their nutritional benefits and significant oil production, particularly in countries like Bangladesh. However, a significant challenge related to these crops is the elevated erucic acid content and increased glucosinolate levels, which limit their suitability for human consumption. Advanced technologies like genome editing and genetic transformation hold considerable promise for addressing these challenges; however, the establishment of effective regeneration systems is a crucial prerequisite for the successful application of these technologies. An effective and reproducible *in vitro* regeneration protocol for *Brassica carinata* A. Br. was developed using MS media supplemented with various concentrations and combinations of BAP, NAA and Kn. The MS medium supplemented with 3.0 mg/l BAP and 0.2 mg/l NAA produced the highest frequency of multiple shoot regeneration from both cotyledonary leaf with petiole and hypocotyl explants. The elongation of the shoot was concurrently optimized in the same media mix. Half-strength MS media supplemented with 0.3 mg/l IBA demonstrated greater efficacy in inducing root formation from excised *in vitro*-derived shoots compared to other hormone supplements. After the successful establishment of well-developed root system, *in vitro*-regenerated plantlets were transferred to soil, resulting in the production of flowers and seeds. The R_1 generation of plants was successfully established in field condition. The *in vitro* regeneration protocol for *B. carinata* developed in this study may serve as a foundation for future plant genetic transformation experiments.

Introduction

The genus Brassica includes numerous economically important vegetable and oilseed crops. Brassica oil seed serves a significant source of edible vegetable oil, protein-rich products, industrial oil, and globally. It is regarded as a one of the major crop in Bangladesh, ranking as the world's third most important vegetable oil after soybean and

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palm (Paul et al. 2020, Hossain et al. 2014). Within the oleiferous Brassica species, *B. carinata*, *B. juncea* and *B. nigra* are commonly designated as mustard, whereas the variants of *B. napus* and *B. rapa* are identified as rapeseed (Yarnell 1956).

Brassica, commonly known as mustard and rapeseed, produces seeds containing 20-25% protein and 40-45% oil (Alim et al. 2020). Due to its omega-3, omega-6 and omega-9 fatty acid content, this cooking oil is considered one of the healthiest options, particularly for individuals with heart conditions (Mustafa et al. 2024). Mustard oil comprises several essential fatty acids required for meeting caloric needs, including palmitic acid, stearic acid, erucic acid, linoleic acid and linolenic acid (Sharafi et al. 2015). It is also rich in vitamin E and natural antioxidants. It also exhibits antibacterial and other therapeutic properties that aid in infection prevention (Mollika et al. 2011). Mustard oil is frequently utilized in Bangladesh for culinary purposes, including cooking, salad dressings and marinating foods prior to frying (Mortuza et al. 2018).

Brassica carinata A. Braun (Ethiopian or Abyssinian mustard) is a promising non-food oilseed crop promoted for renewable fuels, protein-rich feed and bio-based products due to its superior oil quality, environmental adaptability and stable yields (Blackshaw et al. 2011, Cardone et al. 2002, Gesch et al. 2015). Its meal is protein-rich (Schulmeister et al. 2019) and it shows greater tolerance to drought, heat, diseases and seed shattering compared to other *Brassica* species (Shivpuri et al. 1997, Raman et al. 2017). However, increasing climate stress may affect its future productivity.

To meet the growing demands for crop improvement, especially under stress-prone environments, modern biotechnological tools such as genetic transformation and genome editing are essential. The foundation of these techniques lies in the establishment of an efficient and reproducible in vitro regeneration system. Previous reports highlight low regeneration efficiency in *B. carinata* (Yang et al. 1991), which limits its genetic enhancement. Hence, developing a reliable regeneration protocol would support transformation and genome-editing approaches such as *Agrobacterium*-mediated gene transfer and CRISPR-Cas9, enabling the introduction of traits like disease resistance, salinity tolerance, and improved oil quality (Velasco et al. 1998, Getinet et al. 1997). Therefore, this study focuses on establishing an efficient regeneration protocol for *B. carinata*, with relevance to its genetic improvement in Bangladesh.

Materials and Methods

The seeds of *B. carinata* used in the experiment were supplied by the Advanced Chemical Industries (ACI), Tejgaon I/A, Dhaka. Seeds underwent surface sterilization through a 30-second immersion in 70% ethanol (v/v), followed by an 8 min exposure to 1% $HgCl_2$ (w/v) and were subsequently rinsed three to five times with sterilized distilled water. The surface-sterilized seeds were inoculated onto MS medium containing 3% sucrose and 0.8% agar to facilitate germination and seedling development. The cultured seeds were maintained in darkness for two days until germination occurred, after which they were

transferred to a growth room with 16 hrs of light and a temperature of 25 ± 2°C. Seedlings aged four to five days served as the source of initial explants. Various explant types, including cotyledonary leaves with or without petioles, hypocotyls and leaf segments, were excised from the seedlings. Isolated explants were cultivated on MS medium supplemented with BAP (1.0, 2.0, 3.0 and 4.0 mg/l), NAA (0.1 and 0.2 mg/l) and Kn (0.5 and 1.0 mg/l), either individually or in combination, for regeneration. Regenerated shoots were sub-cultured to fresh medium every 2 weeks to ensure ongoing growth. Elongated shoots were cultivated on rooting medium to facilitate root production. To enhance root development, 2-3 cm long shoots were cultured on half-strength MS medium with different concentrations of IBA. Once the plantlets developed a sufficiently robust root system, they were transplanted into small pots filled with autoclaved sterile soil. Pots were covered with transparent perforated polythene bags and maintained in a growth room for one week. Plantlets were acclimatized to the natural environment following appropriate hardening procedures.

Results and Discussion

To establish an efficient and reproducible *in vitro* regeneration system, various concentrations and combinations of BAP, NAA and Kn were utilized in MS for the initiation and development of multiple shoots from *B. carinata*. Cotyledonary leaf with petiole and hypocotyl demonstrated the highest response in terms of both the percentage of shoot regeneration and the number of shoots per explant among the various explants utilized. This study demonstrated that MS medium supplemented with 3.0 mg/l BAP and 0.2 mg/l NAA yielded optimal results for callus induction and shoot regeneration. The maximum number of shoots per explant and the percentage of shoot regeneration were recorded in the identical media composition. The shoot regeneration percentage was 87.5% and the number of shoots per explant ranged from 5 to 8 in this species (Table 1). While the optimal responses for callus induction and shoot regeneration were observed with 3.0 mg/l BAP and 0.2 mg/l NAA, satisfactory results were also achieved with 2.0 mg/l BAP and 0.2 mg/l NAA. Zhao et al. (2021) reported high-efficiency shoot regeneration from cotyledon explants of *B. rapa*. Mollika et al. (2011) previously reported effective responses in two varieties of *B. juncea* regarding shoot development when cultured on MS media supplemented with 2.0 mg/l BAP, 0.2 mg/l NAA and 0.5 mg/l Kn. In contrast, *B. campestris* exhibited optimal shoot development with 3.0 mg/l BAP and 0.2 mg/l NAA. George and Rao (1980) noted that maximum regeneration from cotyledon explants in *B. juncea* occurred with a combination of BAP and NAA, rather than with BAP only. This observation is corroborated by Hachey et al. (1991), who reported effective regeneration in *B. campestris* using BAP in conjunction with NAA. Shoot primordia originated from the cut end of the petiole of the cotyledonary leaf explant. The use of cotyledonary leaves without petioles as explants resulted in a minimal shoot regeneration rate of 1.5 to 8.0%. The presence of the petiole is essential for shoot regeneration when cotyledons serve as explants.

Table 1. Effects of BAP, NAA and Kn on shoot regeneration from cotyledonary leaf with petiole (CP) and hypocotyl (H) explants of *B. carinata.**

Explants	Hormonal supplements (mg/l)			No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean no. of shoots / explant
	BAP	NAA	Kn				
CP	1.0	0.1	-	60	33.30	22-25	1.00
H				60	25.00	-	-
CP	2.0	0.2	-	60	77.50	15-18	4.50
H				60	50.00	-	3.0
CP	3.0	0.1	-	60	63.33	9-15	5.33
H				60	46.50	32-36	3.66
CP	3.0	0.2	-	60	**87.50**	8-12	7.25
H				60	**60.00**	25-30	4.00
CP	4.0	0.2	-	60	73.50	10-18	2.25
H				60	56.50	30-34	1.00
CP	3.0	0.2	0.5	60	25.50	12-16	2.50
H				60	16.60	28-32	1.00
CP	3.0	0.2	1.0	60	35.00	10-15	3.00
H				60	30.50	25-30	1.25

*MS was used in all media combinations

The hypocotyl exhibited the second highest percentage of shoot development, following the cotyledonary leaf with petiole (Fig. 1D). This study reports a 60% shoot regeneration from hypocotyls, with the number of shoots per explant ranging from 2 to 4. Yang et al. (1991) observed a high frequency of plant regeneration from hypocotyl explants in *B. carinata* using a medium that included 2.0 mg/l BAP and 0.01 mg/l NAA. Tang et al. (2003), Zhang et al. (2006) and Khan et al. (2010) identified hypocotyl as an effective explant for shoot regeneration in *Brassica* spp. The regeneration process of Brassica species is significantly influenced by explant age, with younger explants typically exhibiting higher regeneration efficiency. Sharma et al. (1990) and Hachey et al. (1991) found that explants from 4-5 day old seedlings resulted in optimal regeneration across several Brassica species. Additionally, George et al. (2004) indicated that explants from 4-day-old seedlings achieved better results than those from older tissues. Malik et al. (2007) found that cotyledonary leaves with petioles and hypocotyls from 5-day-old seedlings demonstrated the greatest regeneration efficiency, in both direct and indirect shoot formation. The results align with the present study, which utilized explants from 4-5-day old seedlings.

B. carinata demonstrated a 60% rooting success rate in this investigation when grown on half-strength MS medium supplemented with 0.3 mg/l IBA (Fig 1F). This finding is consistent with earlier research indicating that low to moderate concentrations of IBA (0.3-1.0 mg/l) effectively promote root development in Brassica spp. (Alam et al. 2013,

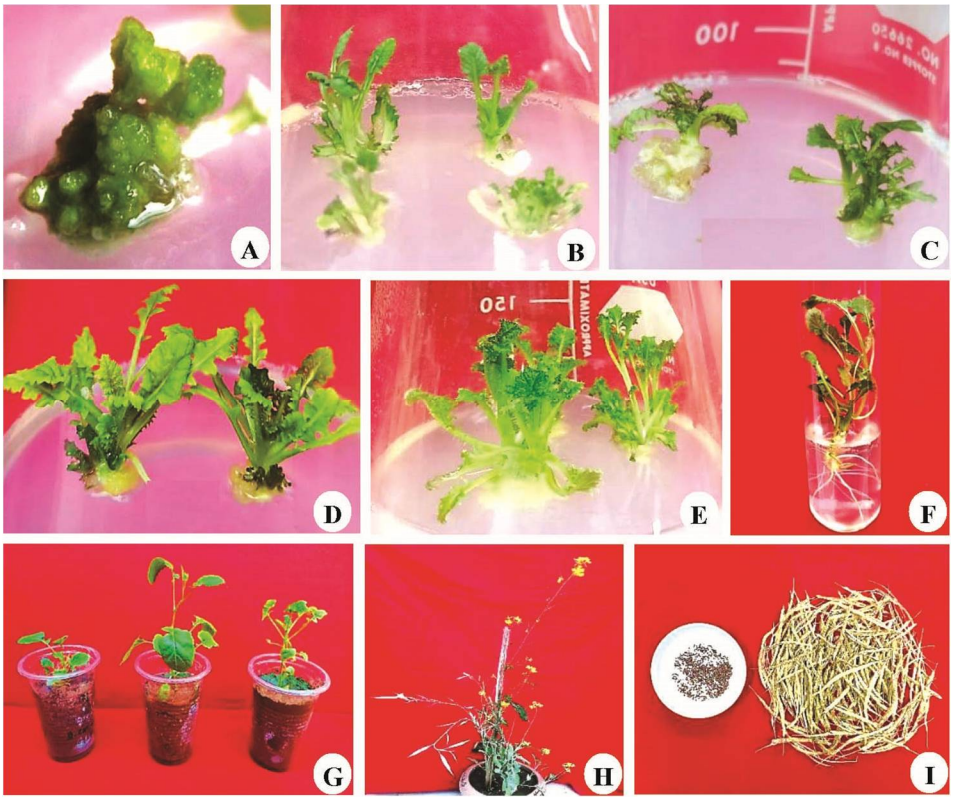

Fig. 1. *In vitro* regeneration of *B. carinata* on MS media supplemented with 3.0 mg/l BAP and 0.2 mg/l NAA: (A) Formation of callus from cotyledonary leaf with petiole, (B & C) Initiation of shoots from hypocotyl and cotyledonary leaf with petiole respectively, (D & E) Multiple shoot formation from cotyledonary leaf with petiole and hypocotyl respectively, (F) Induction of roots on half strength MS media supplemented with 0.3 mg/l IBA, (G) Regenerated plantlets transferred to soil in small plastic pots containing autoclaved soil, (H) Flowers and pod formation in *in vitro*-derived shoots and (I) Harvested seeds from mature pods developed from *in vitro* raised plants.

Goswami et al. 2020). *In vitro*-derived shoots were found to produce *in vitro* flowers on regeneration media (Fig 1E). However, *in vitro* grown flowers were smaller than those grown *in vivo*, which indicates that regenerated shoots may capable of producing flowering hormones through synthesis within their tissues. Comparable results regarding *in vitro* flowering were documented in *B. campestris* by Verma and Singh (2007) and Mollika et al. (2011), as well as in cauliflower by Vandana et al. (1995). After the establishment of adequate root systems, the plantlets were transferred to small plastic pots filled with a soil mixture (Fig. 1G). The acclimatization process is crucial as plantlets progressively adapt to external conditions, including fluctuations in humidity, light and temperature (Jana et al. 2007). The survival rate of the transplanted plantlets was 100%. Flowering occurred between 30 and 40 days, with continuous flowering observed throughout the season (Fig. 1H). Seed formation occurred after 1.5 to 2 months and was

harvested from *in vitro* regenerated plants (Fig. 1l). The R_1 generation seeds demonstrated a germination rate of 92%, comparable to that of the mother plant (Fig. 2). Flower buds appeared after 4 weeks, with complete flowering achieved by 7 to 8 weeks. Siliques formed post-pollination and seeds were harvested following maturation.

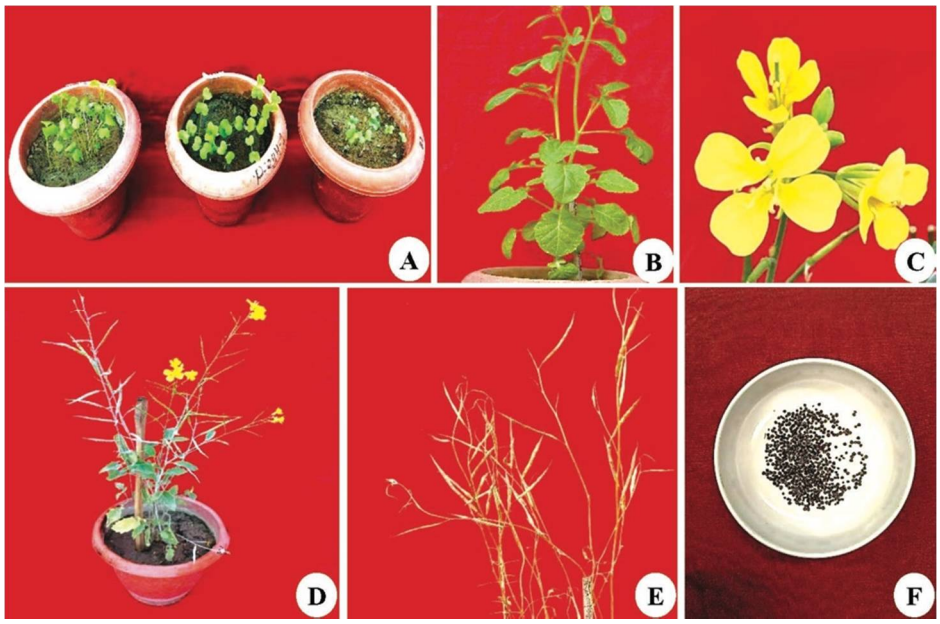

Fig. 2. Establishedment of R_1 generation: (A) Development of R_1 seedlings in large clay pots, (B) One of the healthy R_1 plantlets, (C) Plants at maximum flowering stage within 7 weeks of plantation, (D) Flowes as well as siliques formation on the plants, (E) Mature siliques of one of the R_1 plants and (F) Seeds developed from one of the R_1 plants.

Over all, the regeneration system developed in this study for *B. carinata* is reliable and holds strong potential for the successful application in the genetic transformation of Brassica species.

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References

Alam SS, Khaleda L and **Al-Forkan** M (2013) An Efficient *in vitro* regeneration system for (*Brassica campestris*) Tori-7. Glob. J. Sci. Front. Res. Bio-Tech. Genet. **13**(2): 2249-4626.

Alim MA, Mahfuza M, Hossain MB, Rahman MA, Mostofa MS and **Rakibul M** (2020) Effects of integrated nutrient management on the yield and yield attributes of mustard varieties. Bangladesh J. Agric. Life Sci. **1**(1): 19-27.

Blackshaw R, Johnson E, Gan Y, May W, McAndrews D, Barthet V, McDonald T and **Wispinski D** (2011) Alternative oilseed crops for biodiesel feedstock on the Canadian prairies. Canadian J. Plant Sci. **91**: 889-896.

Cardone M, Mazzoncini M, Menini S, Rocco V, Senatore A, Seggiani M and **Vitolo S** (2003) *Brassica carinata* as an alternative oil crop for the production of biodiesel in Italy: Agronomic evaluation, fuel production by transesterification and characterization. Biomass & Bioenergy **25**(6): 623-636.

George L and **Rao PS** (1980) *In vitro* regeneration of mustard plants (*Brassica juncea* var. RAI 5) on cotyledon explants from non-irradiated, irradiated and mutagen-treated seed. Ann. Bot. **46**(1): 107-112.

Gesch R, Isbell T, Oblath E, Allen B, Archer D, Brown J, Hatfield J, Jabro J, Kiniry J, Long D and **Vigil M** (2015) Comparison of several *Brassica* species in the north central US for potential jet fuel feedstock. Ind. Crops Prod. **75**: 2-7.

Getinet A, Rakow G, Raney JP and **Downey RK** (1997) Glucosinolate content in interspecific crosses of *Brassica carinata* with *B. juncea* and *B. napus*. Plant Breeding. **116**(1): 39-46.

Goswami B, Hoque MI, Khan S and **Sarker RH** (2020) *In vitro* regeneration of three varieties of *Brassica campestris* L. grown in Bangladesh. BJSIR. **55**(3): 181-188.

Hachey JE, Sharma KK and **Moloney MM** (1991) Efficient shoot regeneration of *Brassica campestris* using cotyledon explants cultured *in vitro*. Plant Cell Rep. **9**(10): 549-54.

Hagos R, Shaibu AS, Zhang L, Cai X, Liang J, Wu J, Lin R and **Wang X** (2020) Ethiopian mustard (*Brassica carinata* A. Braun) as an alternative energy source and sustainable crop. Sustainability. **12**(18): 7492.**Hussain S**, **Rasheed A**, **Latif M**, **Mahmood TARIQ** and **Naqvi SS** (2014) Canola (*Brassica napus* L.) regeneration and transformation via hypocotyl and hypocotyl derived calli. Sarhad J. Agric. **30**(2): 165-172.

Jana P, Helena S, Daniel H, Šárka S (2007) Acclimation of plantlets to ex vitro conditions: effects of air humidity, irradiance, CO_2 concentration and abscisic acid. *Acta Horticulturae.* **748**: 29-38.

Khan MMA, Robin ABM AHK, Nazim-Ud-Dowla MAN, Talukder SK and **Hassan L** (2010) *In vitro* regeneration potentiality of oil seed *Brassica* genotypes in differential growth regulator. Bangladesh J. Agric. Res. **33**(2): 189-191.

Kumar S, Seepaul R, Mulvaney MJ, Colvin B, George S, Marois JJ, Bennett R, Leon R, Wright DL and **Small IM** (2020) *Brassica carinata* genotypes demonstrate potential as a winter biofuel crop in South East United States. Ind. Crops Prod. **150**: 112353.

Lin L, Allemekinders H, Dansby A, Campbell L, Durance-Tod S, Berger A and **Jones PJ** (2013) Evidence of health benefits of canola oil. Nutrition reviews **71**(6): 370-385.

Malik MR, Wang F, Dirpaul JM, Zhou N, Polowick PL, Ferrie AMR and **Krochko JE** (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus.* Plant Phys. **144**(1): 134-154.

Mollika SR, Sarker RH and **Hoque MI** (2011) *In vitro* Plant Regeneration in *Brassica* spp. Plant Tiss. Cult. Biotech. **21**(2): 127-134.

Morinaga T (1934) Interspecific hybridization in *Brassica*. The cytology of F_1 hybrid of *Brassica juncea* and *Brassica nigra*. Cytologia. **6**: 62-67.

Mortuza MG, Dutta PC and **Das ML** (2006) Erucic acid content in some rapeseed/mustard cultivars developed in Bangladesh. J. Sci. Food Agric. **86**: 135-139.

Mustafa, HSB, Zulfqar M, Ejaz-ul-Hasan TM, Qamar H, Hassan M, Shahzad S, Riaz M, Mohyo-ud-Di A, Nawaz A, Gill QAT and **Altaf M** (2024) A High-Yielding Rapeseed Cultivar Enriched With Omega-3 And Omega-9 Fatty Acids. Plant Bulletin. **3**(1): 10-18.

Paul M, Islam T, Hoque MI and **Sarker RH** (2020) Analysis of genetic diversity in oilseed Brassica germplasm through ISSR markers and isozyme profiling. Bangladesh J. Bot. **49**(1): 147-158.

Raman R, Qiu Y, Coombes N, Song J, Kilian A and **Raman H** (2017) Molecular diversity analysis and genetic mapping of pod shatter resistance loci in *Brassica carinata* L. Front. Plant Sci. **8**: 1765.

Schulmeister TM, Ruiz- Moreno M, Silva GM, Garcia- Ascolani M, Ciriaco FM, Lamb GC, Dubeux JCB and **Dilorenzo N** (2019) Evaluation of *Brassica carinata* meal as a protein supplement for growing beef heifers. J. Animal Sci. **97**(10): 4334-4340.

Sharafi Y, Majidi MM and **Goli SAH** (2015) Oil content and fatty acids composition in Brassica species. Int. J. Food. Prop. **18**(10).

Sharma KK, Bhojwani SS and **Thorpe TA** (1990) Factors affecting high frequency differentiation o f shoots and roots from cotyledon explants of *Brassica juncea* (L.). Czern. Plant Sci. 66:

Shivpuri A, Chipa H P, Gupta RBL and **Sharma KN** (1997) Field evaluation of mustard genotypes against white rust, powdery mildew and stem rot. Ann. Arid Zone **36**(4): 387.

Tang GX, Zhou WJ, Li HZ, Mao BZ, He ZH and Yoneyama K (2003) Medium, explant and genotype factors influencing shoot regeneration in oilseed *Brassica* spp. J. Agron. Crop Sci. **189**: 351-358.

Vandana AK, Kumar A and **Kumar J** (1995) *In vitro* flowering and pod formation in cauliflower (*B. oleracea* var. botrytis). Curr. Sci. **69**(6): 543-545.

Velasco L, Fernandez- Martinez JM and **Haro AD** (1998). Increasing erucic acid content in Ethiopian mustard through mutation breeding. Plant Breeding **117**(1): 85-87.

Verma R and **Singh RR** (2007) Regeneration and *in vitro* lowering in *Brassica campestris* (L.) Var. Bhavani. Nature **5**(1): 21-24.

Yang MZ, Jia SR and **Pua EC** (1991) High frequency of plant regeneration from hypocotyl explants of *Brassica carinata*. Plant Cell Tiss. Org. Cult. **24**: 79-82.

Yarnell SH (1956) Cytogenetics of the vegetable crops. II. Crucifers. The Bot. Rev. **22**: 81-166.

Zhang Y, Xu J, Han L, Wei W, Cong L and **Chai T** (2006) Efficient shoot regeneration and *Agrobacterium*-mediated transformation of *Brassica juncea*. Plant Mol. Bio. Rep. **24**(2): 255a-255i.

Zhao Y, Huang S, Zhang Y, Shi F, Liu X, Du S and **Feng H** (2021) Establishment of an efficient shoot regeneration system *in vitro* in *Brassica rapa*. *In vitro* Cell. Dev. Biol. Plant. **57**: 977-986.

Zhou WJ, Zhang GQ, Tuvesson S, Dayteg C and **Gertsson B** (2006) Genetic survey of Chinese and Swedish oilseed rape (*Brassica napus* L.) by simple sequence repeats (SSRs). Genet. Res. Crop Evol. **53**(3): 443-447.

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