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An Efficient in Vitro Regeneration from Hypocotyl and Cotyledon Explants of Brassica Juncea (L.) Coss

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Abstract: *In vitro studies of Brassica juncea is indispensable for evaluating its response to different biotic and abiotic stresses and its improvement through biotechnological techniques. In order to enrich its genetic resources; an efficient method of shoot regeneration is required. Culture media explants and genotypes are major factors that influence the regeneration of plant. In this assay hypocotyls and cotyledonary petioles obtained from in vitro grown seedlings were used as explants for indirect regeneration of four Brassica juncea genotypes namely RH 0406, RH 0555, RH 0749 and RH 0832 to observe their regeneration potentiality. Single and combinatorial effects of plant growth regulators (PGRs) and additives were observed.*

Varying concentrations of hormones growth regulators were supplemented on MS medium were found to change efficiency of callus, shoot and root formation. Among the treatments the highest percentage of shoot induction 83.33% was observed on MS+Kin (2.5 mg/l)+NAA (0.2 mg/l) in cotyledons of RH0832 and hypocotyls of RH 0749 showed the highest percentage of callus induction (83.33%). Followed by MS+BAP (2.5 mg/l)+NAA (0.2 mg/l) that shows 75.55% shoot induction in RH 0749 cotyledons. Hypocotyls were found efficient (70-80%) for callus production while their response for shoots formation was slower as more number of days was taken by hypocotyls to produce shoots. Half strength MS and MS+0.2 mg/l IAA gave the best results for root induction.Based on our study, hypocotyls and roots could be the preferred explants when more and vigorous callus formation is required, while cotyledons could be the preferred explants where regeneration is needed such as in genetic transformation experiments.

1. INTRODUCTION

Since the last few decades, oil crops have drawn the attraction of the world agriculture and associated industries because of their economic interest and food value. Refined agronomic practices and plant breeding techniques have opened new doors that have significantly increased oil crop production.Among them, *Brassica* is one of the most important sources of edible vegetable oil, industrial used oil and protein-rich product in the world. Oil seed *Brassica* ranks third after soybean and palm oil in the global production (Canola Council of Canada 2006). In this subcontinent three species of *Brassica*, namely, rapeseed, *Brassica rapa* (Syn. *Brassica campestris* L.), mustard, *B. juncea* (L.) Czern and Coss and *B. napus* L. belonging to Brassicaceae (Cruciferae) are cultivated for the production of oil.

Brassica juncea (L.) is a major oilseed crop of Indian sub-continent. Although, oilseed Brassica are grown over 15% of arable land in India but their productivity was considerably hindered mainly insect pests and diseases. Cultivated Brassica normally do not have any inherent resistance genes in them through conventional breeding faces its own disadvantages.Conventional breeding programmes alone were not successful enough in *Brassica* due to high degree of segregation upon cross-pollination and unavailability of suitable wild germplasm. Enrichment of genetic variability through mutation, somaclonal variation, and protoplast fusion contributed only a little in the production of disease and pest resistant plants to overcome incompatibility barriers as well as plants with better agronomic characters in *Brassica* spp. In this regard, *in vitro* regeneration and transformation have prospects to fulfil breeding needs. Tissue culture or *in vitro* micropropagation technique has been applied for *Brassicas* for a long time observed by Ali *et al* (2007)and John *et al* (1991).

A considerable amount of research has already been undertaken in this direction, and rapeseed has been exploited for genetic engineering purposes. Various protocols for in vitro regeneration and transformation of Brassica spp. have been reported, which gave varying regeneration responses from different explants, such as cotyledons, cotyledonary node petioles (82%), hypocotyls (20-23%) and stem segments (56-100%) (Guruprasad *et al.* 2011). In vitro morphogenesis in *B. juncea* has been studied and various tissue explants such as

hypocotyls (Wang *et al*. 200 0), c otyledons (Hachey *et al.* 1991).

The present w ork w as c onducted t o establish a r eproducible protocol f or callus induction and r egeneration of 4 r apeseed (*Brassica juncea*) ge notypes viz., RH 0 406, RH 05 55, R H 0749 and RH 0832 using hypocotyls and cotyledons as explant source. The objectives were t o s elect t he b est m edia combination for callusing and regeneration and to find out the best responsive genotypes on selected media.

2. MATERIAL AND METHODS

The seeds of *Brassica juncea* genotypes RH 406, RH 555, RH 0749 a nd R H 0832 were obtained from t he r esearch a rea o f Oilseeds Section, Department of Genetics and Plant Breeding, CCS Ha ryana Agricultural U niversity, H isar. T heexplants collected f rom *in vitro* grown seedlings u sed f orthe experiments we re: hy pocotyls a nd c otyledons. E xplants from 3-5 da ys ol d s eedlings were t aken. The ba sal a nd a pical portions o f h ypocotyls and roots w ere di scarded and the middle p art was cu t into 5 mm l ong s egments. Two e xplants from each cotyledon of a seedling were prepared by cutting in the middle.

The s eeds were f irst washed w ith t ween 20 a nd t hen s urface sterilized with mercuric chloride (0.1%) for 5-7 mins followed by thr ee w ashings w ith s terilized d istilled water i n l aminar hood. Sterilized s eeds w ere aseptically inoculated o n H alf strength M S (Murashige & Skoog, 1 962) medium c onsisting of 2% sucrose, solidified w ith 0. 8% agar w as used for s eed germination a nd s eedling growth w hereas, MS m edium comprising i norganic s alts a nd vi tamins s upplemented with 3%, sucrose, di fferent concentrations of a uxins (indole-3-acetic acid, α-naphthalene a cetic a cid) a nd cytokinins (BAP, TDZ and K inetin) and solidified w ith 0. 8% a gar was us ed to induce cal li a nd s hoots production All m edia w ere s terilized by a utoclaving a t 121^{o}C a nd 15 PPS^{-1} for 1 5 minutes. Initiation of roots was tried on mediasupplemented with NAA and IBA. After c ulturing e xplants on M S m edium supplemented w ith di fferent c oncentrations of PGRs, i n g lass Petri di shes, cultures w ere i ncubated a t 2 5oC under 16 h light/8 h dark c onditions a nd o bserved regularly f or c allus initiation a nd/or shoot f ormation. Pictures o f t he e xplants forming c allus a nd s hoots, r egenerated shoots a nd rooted shoots were taken The p lantlets w ithsufficient rooting system were taken out of the culturevessels and the roots were washed under tap water. T he*in vitro* grown r ooted pl ants were t hen transferred i ntosmall pot s. Hardening w as c arried out by periodicalexposure of the plants to natural environment.

3. RESULTS AND DISCUSSIONS

Different c oncentrations o f BAP, NAA, TDZ a nd K n were used in MS to determine the optimum media composition for initiation a nd de velopment of m ultiple shoots from thr ee varieties o f *Brassica* used in t his i nvestigation. Among t he explants us ed, c otyledons a nd hypocotyls, c otyledons were found t o be t he m ore responsive i n t erms o f percentage o f shoot regeneration as well as the number of shoots per explant in a ll t hevarieties t ested. G eorge a nd Ra o (1980) o bserved maximum r egeneration from c otyledon e xplants in *B. juncea* with BAP and NAA rather than BAP alone. Tang et al. (2003) observed s imilar r esponse towards s hoot regeneration i n both between and within *Brassica* species.

Cotyledon explants

The cotyledons (fig. 1) e xcised f rom 5 -day r aised s eedlings were c ultured o n M S m edium s upplemented with different concentrations a nd c ombinations of g rowth regulators. After 1-2 weeks of culture, cotyledons began to expand in size and showed c allusing a t t he petiolar e nd. S hoots i nitiated and began to proliferate within 2-4 weeks (Fig.2). In genotype RH 406 a nd R H 832, hi ghest per c ent shoot r egeneration was observed o n M S m edium s upplemented wi th Kin (2.5 mg/l)+NAA (0.2 m g/l) (67.83+2.37) an d (83.33+5.09) respectively. L owest response wa s observed o n M S m edium fortified w ith IAA (1 mg/l) (9.65+5.34) a nd (0 0+00). In genotype RH 55 5, hi ghest per cent shoot r egeneration w as obtained o n M S m edium s upplemented w ith B AP (2.5 m g/l) (75.56+5.88) a nd l owest on MS m edium c ontaining IAA (1 mg/l) and Kin (2.5 m g/l)+NAA (0 .2 m g/l) (2.22+2.22) an d (41.11+8.67). I n ge notype R H 749, hi ghest per cent shoot regeneration was obtained on MS medium supplemented with TDZ (2.5 m g/l) (71.11+4.84) a nd low est on MS m edium containing I AA (1 m g/l) and Kin (5 .0 m g/l)+NAA (0 .2 mg/l)(1.11+1.11) a nd (34.44+2.94). MS m edium supplemented wi th BAP (2.5 mg/l)+NAA (0.2 m g/l) y ielded highest s hoots pe r e xplant i n genotypes R H 406 (11.4+1.5), BAP (2.5 mg/l) in ge notype R H555 (11.4+0.812), T DZ (2.5 mg/l) in genotype R H555 (11.2+1.319) a nd B AP (5.0 mg/l)+NAA (0.2 m g/l) i n ge notype R H832 (10+0.707) . P er cent s hoot f ormation a nd shoots pe r e xplant i n c ultured cotyledons f or a ll ge notypes o n va rious m edia c ombinations are presented in table 2.

Hypocotyl explants

Hypocotyl (Fig.) explants excised from 5-day raised seedlings were c ultured o n M S m edium s upplemented with different concentrations a nd c ombinations o f g rowth r egulators a s shown i n Table 2 . The c ultured hy pocotyl e xplants s howed initial s welling w ithin 1 -2 we eks. H ypocotyls e xplants responded e ither b y f orming c alli o r m ultiple s hoots formation. They began to form calli within 2-4 weeks (Fig.7). Explants s howed different be havior on di fferent m edia combinations. C ompared t o c otyledon e xplant, hy pocotyls showed p oor regeneration a s a fter i noculation c reamy whi te coloured callus was formed at both cut edges of hypocotyl and with the increase of time most of the explants became brown and f inally di ed. It m ay be m entioned t hat hy pocotyl as a

suitable explant for regeneration of shoots was reported Zhang et al. (2006) and Khan et al. (2010).

Out of 10 media combinations tried for regeneration experiments, highest per cent shoot formation was observed on MS medium supplemented with BAP (2.5 mg/l)+NAA (0.2 mg/l) i.e. 65.34+3.16 in genotype RH 406, whereas highest shoots per explant were observed on MS medium supplemented with Kin (2.5 mg/l)+NAA (0.2 mg/l) i.e. 4.8+0.66. In genotype RH 555,BAP (2.5 mg/l) showed maximum shoot regeneration i.e. 44.44+4.84. In genotype RH 749, hhypocotyls showed better response with Kin (2.5 mg/l) i.e. 83.33+5.774 and in genotype RH 832 Kin (2.5 mg/l)+NAA (0.2 mg/l) showed maximum regeneration i.e. 71.11+4.00.Whereas highest per cent shoot formation was observed on MS medium containingKin (2.5 mg/l)+NAA (0.2 mg/l) is 4.8+0.66 in RH 406, BAP (2.5 mg/l) showed maximum i.e. 2.6+0.51 in RH 555 and 3.4+0.81 in RH 832. TDZ (2.5 mg/l) showed maximum regeneration 11.2+1.02 in RH 749. Per cent shoot formation and shoots per explants in cultured hypocotyls for both the genotypes on various media combinations are presented in Table 2.

In the present investigation a considerable variation in shoot regeneration from cotyledon explants was observed in all the varieties used. Among the three varieties used, RH 0832were most responsive based on the above mentioned observation.Highest number of shoots per explants was in RH406 and RH 555. Moreover, the day required for shoot regeneration was less than other varieties were in RH 832. *Brassica* is highly genotype dependent and genotype specificity is a limiting factor in *Brassica* tissue culture and regeneration, which severely limits the germplasm that can be manipulated or improved It was also observed by Cardoza and Stewart(2004).

It was also found during the present experiment that, regeneration is dependent on the age of the explants, it was reported that young explants have been given better results than older explants. Explants of 4 - 5 days old seedlings showed best response towards both direct and indirect shoot regeneration. Most researchers have found that explants excised from 4 - 5 days old seedlings gave optimal regeneration rates in different *Brassica* spp. (Sharma et al. 1990, Hachey et al. 1991).

Table 2: Effect of phytohoromone on shoot regeneration from cotyledon explants of four genotypes of *Brassica juncea*

Genotypes	Hormonal supplements (mg/l)				No. of explants inoculated	% of responsive explants	Days to shoots initiation	Mean no. of shoots/ explant
	BAP	NAA	Kn	TDZ				
RH 0406	2.5	-	-	-	30	63.33 +5.09	15-20	10.6+1.0
	1.0	0.2	-	-	30	66.66+5.09	17-22	8.8+0.9
	2.5	0.2	-	-	30	58.89+6.18	9-16	11.4+1.5
	5.0	0.2	-	-	30	54.44+16.81	11-18	8.2+1.5
	-	-	2.5	-	30	45.55+7.77	15-19	9.6+2.3
	-	0.2	1.0	-	30	47.77+9.68	18-24	7.6+1.5
	-	0.2	2.5	-	30	56.66+9.62	09-15	8.8+0.9
	-	0.2	5.0	-	30	65.55+4.84	10-17	6.6+1.1
	-	-	-	2.5	30	72.22+7.77	10-18	8.2+2.0
	-	-	-	1	30	4.443+2.94	22-25	1.4+0.7
RH 0555	2.5	-	-	-	30	75.55+5.88	17-20	11.4+0.81
	1.0	0.2	-	-	30	62.22+4.84	11-16	10.0+1.41
	2.5	0.2	-	-	30	72.22+6.75	18-24	9.0+1.00
	5.0	0.2	-	-	30	68.89+6.75	09-15	6.6+1.07
	-	-	2.5	-	30	57.78+4.84	15-20	6.6 +1.327
	-	0.2	1.0	-	30	46.66+8.81	10-17	3.2 +0.583
	-	0.2	2.5	-	30	41.11+8.67	15-19	6.6 +0.784
	-	0.2	5.0	-	30	53.33+8.81	18-23	7.2+0.8
	-	-	-	2.5	30	65.55+1.11	11-18	8.2+1.068
	-	-	-	1	30	2.22+2.22	17-22	1+0.632
RH 0749	2.5	-	-	-	30	68.89+4.84	9-16	6.0+1.30
	1.0	0.2	-	-	30	46.66+5.09	11-15	5.6+0.92
	2.5	0.2	-	-	30	63.33+ 3.84	15-18	4.6+0.82
	5.0	0.2	-	-	30	49.99+ 8.81	12-15	3.2+0.86
	-	-	2.5	-	30	70+3.85	10-17	3.6+0.67
	-	0.2	1.0	-	30	34.44+2.9	14-16	1.6+0.4
	-	0.2	2.5	-	30	55.55+ 6.18	11-14	5.6+0.51
	-	0.2	5.0	-	30	37.78+4.84	12-17	2.0+0.63
	-	-	-	2.5	30	71.11+4.84	10-16	11.2+1.31

	-	-	-	1	30	1.11+1.11	22-25	0.4+0.4
RH 0832	2.5	-	-	-	30	50+5.77	15-18	8.2+1.2
	1.0	0.2	-	-	30	45.55+7.28	9-16	9.6+1.03
	2.5	0.2	-	-	30	71.11+ 4.84	12-17	7.2+1.02
	5.0	0.2	-	-	30	47.78+4.84	10-15	10 +0.70
	-	-	2.5	-	30	75.55 +4.84	11-16	3.8+0.91
	-	0.2	1.0	-	30	66.67+5.77	9-14	2.8+0.73
	-	0.2	2.5	-	30	83.33+5.09	11-15	3.8+0.73
	-	0.2	5.0	-	30	36.66+3.84	14-17	4.8+0.66
	-	-	-	2.5	30	46.66+7.69	12-15	6.6+1.07
	-	-	-	1	30	00+00	00-00	0.0+0.0

Table 2: Effect of different combinations of phytohoromoneson shoot regeneration from hypocotyle explants of four genotypes of Brassica juncea

Genotypes	Hormonal supplements (mg/l)				No. of explants inoculate	% of responsive explant	Days to shoots initiation	Mean no. of shoots/ explant
	BAP	NAA	Kn	TDZ				
RH 0406	2.5	-	-	-	30	73.33+5.77	30-35	1.8+0.37
	1.0	0.2	-	-	30	68.89+4.84	30-32	03+0.54
	2.5	0.2	-	-	30	58.89+14.69	33-36	2.8+0.58
	5.0	0.2	-	-	30	57.78+4.84	28-32	3.8+0.66
	-	-	2.5	-	30	67.77+14.69	30-34	3.2+0.73
	-	0.2	1.0	-	30	46.66+5.09	28-33	2.8+0.37
	-	0.2	2.5	-	30	64.44+ 9.09	30-32	4.8+0.66
	-	0.2	5.0	-	30	41.11+4.00	30-34	2.0+0.58
	-	-	-	2.5	30	53.33 +6.93	33-36	3.0+0.44
	-	-	-	1	30	1.11+ 1.11	34-38	0.6+0.4
RH 0555	2.5	-	-	-	30	44.44+4.84	30-33	2.6+0.51
	1.0	0.2	-	-	30	33.33+3.84	31-34	1.8 +0.374
	2.5	0.2	-	-	30	42.22 +11.6	33-36	2.2 +0.583
	5.0	0.2	-	-	30	27.77+4.00	28-33	1.2 +0.374
	-	-	2.5	-	30	34.44+4.00	30-34	0.8 +0.374
	-	0.2	1.0	-	30	43.33+5.77	32-35	0.6+0.4
	-	0.2	2.5	-	30	43.33+10.18	33-36	1.2 +0.583
	-	0.2	5.0	-	30	28.89+6.75	28-32	1.4 +0.748
	-	-	-	2.5	30	40.0 +7.69	32-34	1.2 +0.583
	-	-	-	1	30	00+00	00-00	00+00
RH 0749	2.5	-	-	-	30	73.3+5.77	30-34	1.6+0.4
	1.0	0.2	-	-	30	60+5.77	28-32	3.6+0.51
	2.5	0.2	-	-	30	78.88+7.28	28-32	3.0+0.70
	5.0	0.2	-	-	30	56.66+8.38	30-34	3.6+0.81
	-	-	2.5	-	30	83.33+5.77	31-34	3.2+0.66
	-	0.2	1.0	-	30	47.78+6.75	33-36	2.8+0.86
	-	0.2	2.5	-	30	56.67+5.77	28-33	10.4+1.12
	-	0.2	5.0	-	30	57.77+7.77	30-32	5.2+0.66
	-	-	-	2.5	30	78.89+ 4.84	32-34	11.2+ 1.02
	-	-	-	1	30	00+00	00-00	0.0+0.0
RH 0832	2.5	-	-	-	30	35.55+2.94	28-32	3.4+0.81
	1.0	0.2	-	-	30	32.22 4.84	28-33	1.8+0.73
	2.5	0.2	-	-	30	47.77+7.79	30-34	3+0.54
	5.0	0.2	-	-	30	35.55+4.84	28-33	2.8+0.86
	-	-	2.5	-	30	58.89+4.84	32-35	2.8+0.58
	-	0.2	1.0	-	30	54.44+6.76	33-36	2.4+0.92
	-	0.2	2.5	-	30	71.11+4.00	30-32	2+0.70
	-	0.2	5.0	-	30	32.22+6.18	31-34	2.6+0.51
	-	-	-	2.5	30	50+3.85	28-32	2.4+0.92
	-	-	-	1	30	0+00	00-00	0.0+0.0

To get a complete plantlet, root formation is an obligatory step. Spontaneous root generation occurs sometimes on MS medium with hormonal supplements for the induction of shoots but plantlets with these roots were not found to be efficient to thrive in soil. Therefore, independent media supplemented with NAA and IBA were used to induce and develop roots. Half strength MS medium with supplementation of 0.2, 0.5 mgL^{-1} IBA and 0.2mg/L NAA in each treatment were used to observe rooting response of regenerated shoots.

During experiment the highest percentage of root induction was observed in MS half strength media in genotype RH 0749 and RH 0555 i.e. 81.13+8.86 and 72.27+8.86 respectively and lowest i.e. 30.775+4.22 was observed in RH 0832 in MS+NAA (0.2mg/L). Among the three treatments half MS medium was found to be the best for root initiation The number of roots decreased with increasing NAA concentrations. This was also observed by Basak et al. (2012)

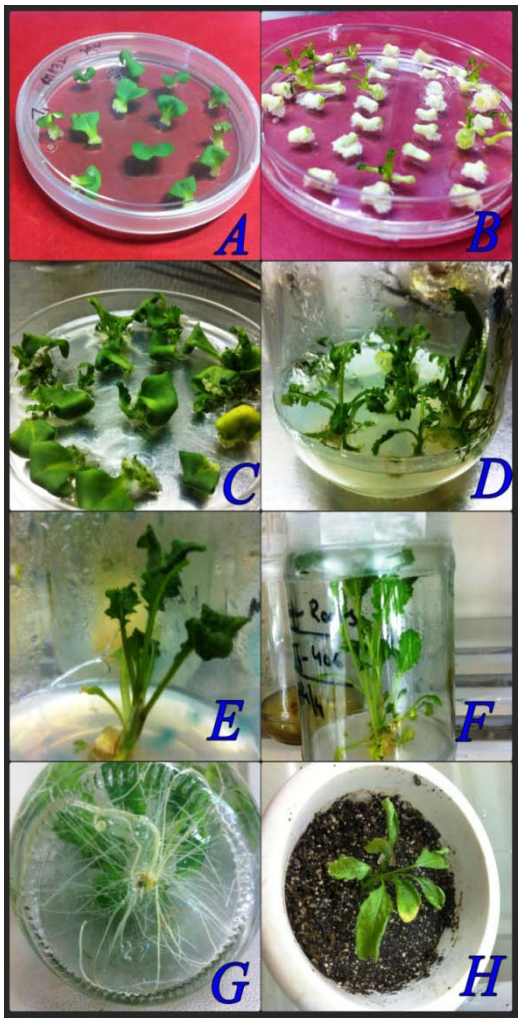

Fig. 1: Callus, Shoots and adventitious roots production by cotyledon and hypocotyls of *Brassica juncea*.

A. Cultured cotyledon explants
B. Callusing in hypocotyl explants.
C. Shoot induction in cotyledon explants
D. Shoot induction from callus of hypocotyl explants.
E. Regenerated plant from hypocotyl.
F. Regenerated plant from cotyledons.
G. Root induction in regenerated plant.
H. Transfer of regenerated plant to pot.

After sufficient development of root system the small plantlets were taken out from the culture vessels without damaging roots. Excess agar around the roots was washed off by running tap water to prevent microbial infection and transplanted in small plastic pots containing sterile soil and coco pit.The pots were then covered with clear polyethylene bag to maintain high humidity conditions and kept in the growth chamber for proper hardening. Gradually the plantlets were adapted to soil and established. Among the four genotypes, RH 0832 produced highest survived plants (52.25%) followed by RH 0406 (40%).

Table 3: Effects of different combinations of phytohoromone in half strength MS medium on root initiation

Sr. No.	Rooting media	percent root formation			
		RH 0406	RH 0555	RH 0749	RH 832
1	MS basal	51.12+6.98	54.96 +4.22	46.90+3.84	43.06+3.84
2	Half strength MS basal	68.05+11.56	72.27+8.86	81.13+8.86	59.189+4.22
3	MS+IBA (0.2mg/L)	59.18+4.22	46.90+3.84	72.27+8.86	54.96+4.22
4	MS+IBA (0.5 mg/L)	54.96+4.22	51.12+6.98	43.06+3.84	46.90+3.84
5	MS+NAA (0.2mg/L)	38.83+6.98	43.06+3.84	34.95+4.22	30.775+4.22

4. CONCLUSIONS

Explants from four-five days old seedlings of four *Brassica* genotypes were cultured on MS medium supplemented with different concentrations and combinations of hormone. Shoot induction was highest in RH 0832 (83.33%) in cotyledons and lowest in genotype RH 0555 (28.89 %) in hypocotyls. The highest number of root was observed in RH 0749 (81.13%) and lowest number was found in RH 0832 (30.77%). The maximum number of roots was observed in ½ MS medium against all the genotypes.In this experiment, *in vitro* regeneration potentiality of four *Brassica* genotypes has been observed and an efficient as well as reproducible protocol for regeneration of the genotypes has been developed using cotyledon and hypocotyls as explants, this protocol can be followed for genetic manipulation for improvement of *Brassica* species.

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