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Characterization of a Collection of *Brassica carinata* Genotypes for *in vitro* Culture Response and Mode of Shoot Regeneration

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ABSTRACT

Brassica carinata, a natural alloploid formed between B. oleracea and B. nigra, is a potential oil crop for the Mediterranean area in which genetic transformation could help to breed. In vitro culture and shoot regeneration are key factors in developing an efficient transformation method in the genus Brassica. However, the studies for in vitro culture and shoot regeneration in B. carinata are limited to only a few genotypes. The aim of this study was to evaluate the in vitro culture response and shoot regeneration in a collection of B. carinata accessions to identify promising genotypes with high shoot regeneration for genetic transformation experiments. Cotyledonary explants from 51 genotypes were cultured in vitro and callus formation and swelling as well as the mode of shoot regeneration evaluated. A highly positive response to in vitro culture, i.e. callus formation or swelling, was observed in all the genotypes tested. Tissue blackening occurred only in eleven genotypes. Parameters like callus formation and swelling and number of shoots per explant were highly variable among genotypes. Fourteen genotypes regenerated only via callus formation, whereas only one regenerated only via swelling. Most genotypes showed a higher percentage of callus formation than swelling. The average number of shoots regenerating per explant among genotypes was the most variable factor measured. Six genotypes regenerated more than 6 shoots per explant via callus phase. These genotypes have been identified as having a high regeneration potential and can be used in genetic transformation via Agrobacterium.

Keywords*: *Mustard*, *Tissue Culture*, *Genotype*, *Cotyledons*, *Swelling*, *Callus

1. Introduction

Rapeseed (*Brassica napus* L.) is the third most important source of vegetable oil in the world with 31 million cultivated hectares in the year 2009 (FAOSTAT, 2011). Modification of the fatty acid composition is currently an important objective of plant breeding of this crop [1] and there is considerable commercial interest in the development of high erucic acid and/or low glucosinolates lines targeted toward industrial end-use and in the development of low (or zero) erucic acid, low linoleic and high oleic lines for food industries.

Ethiopian mustard, *B. carinata* A. Braun (BBCC, 2n = 4x = 34), is a natural alloploid from *B. oleracea* L. (CC, 2n = 2x = 18) and *B. nigra* (L.) W.D.J. Koch, (BB, 2n = 2x = 16) which has several agronomical important traits such as nondehiscent siliques and a much more developed and aggressive root system than *B. napus*. It is resistant to a wide range of diseases and pests and is tolerant to many abiotic stresses [2-4], which makes it a suitable candidate as a winter crop in the Mediterranean countries.

Plant transformation systems have been developed for many economically important species of the genus *Brassica* such as *B. napus* [5], *B. oleracea* [6], *B. juncea* [7], *B. rapa* [8], *B. nigra* [9] and *B. carinata* [10]. This technology enables us to obtain transgenic plants with modified agronomic traits. Many genetic improvements, such as herbicide tolerance, improved oil quality and production of pharmacological and industrial products, have been achieved by genetic transformation in the *Brassica* species. For example, *B. napus* seeds containing high levels of gamma-linoleic acid were obtained by the introduction of δ12-desaturase genes from the fungus *Mortierella alpine* [11]. In addition, *B. napus* genotypes were developed for sulfonylurea resistance and bro-

moxynil resistance by Blackshaw *et al.* [12] and Zhong [13] respectively. In *B. carinata*, Jadhav *et al.* [14] increased the level of erucic acid in the seeds by co-suppression and antisense repression of the endogenous *FAD*2 gene encoding the oleate desaturase FAD2.

In vitro regeneration is a key factor in developing an efficient transformation method in plants. *In vitro* regeneration in *Brassica ssp.* is highly genotype-dependent as reported in previous studies for *B. napus* [15,16], *B. juncea* [17], *B. rapa* [18] and *B. oleracea* [19]. In addition, Dietert *et al.* [20] compared 6 species of the genus *Brassica* for callus growth and plant regeneration and reported a high influence of the genotype, with as much inter-cultivar as inter-species differences in the response to the in vitro culture. However, the available information for the genotype variability for *in vitro* culture and shoot regeneration in *B. carinata* is limited to a small number of genotypes. This genotype-dependence of the *in vitro* culture is a limiting factor for the application of genetic engineering to a wide number of genotypes. For that reason, it is important to identify highly regenerant genotypes that can be used in transformation via *Agrobacterium tumefaciens.*

This study aims to evaluate the *in vitro* culture response, *i.e.* callus formation and swelling, and the mode of shoot regeneration of a collection of *B. carinata* accessions to identify promising genotypes with high regeneration potential.

2. Materials and Methods

2.1. Plant Material

Fifty-one lines of *B. carinata* supplied by the Regional Plant Introduction Station (Iowa State University, Iowa, U.S.) (**Table 1**) were evaluated for *in vitro* culture and shoot regeneration response. For comparison, two lines were used as controls; a *B. carinata* DH line (BC71), which showed a good response to microspore culture [21] and a *B. oleracea* DH line (AG1012) provided by the Department of Crop Genetics (John Innes Centre, Norwich, UK) which was selected for its high regeneration and transformation potential [22].

2.2. *In vitro* Culture

Seeds were surface sterilized with 100% ethanol for 2 min and with a 15% sodium hypochlorite solution containing 0.1% Tween-20 for 15 min. Then, seeds were washed 3 times with sterile distilled water and air-dried in flow hood chamber. Seeds were germinated in 15 x 90 mm Petri dishes containing 25 ml of germination medium, which consisted of 4.3 g/l MS salts [23] plus 3% sucrose and 0.8% phytoagar at pH 5.8. After autoclaving,

Table 1. List of *Brassica. carinata* genotypes used in the experiment supplied by the Regional Plant Introduction Station (Iowa State University, Iowa, U.S.). Genotypes are ordered by accession number.

Code number	Accession number	Origin		
		Country	State	Plant name
1	193459	Ethiopia	Shewa	*Unknown*
2	193460	Ethiopia	Harer	NU 51639
3	193467	Ethiopia	Shewa	NU 51640
4	193759	Ethiopia	Shewa	*Unknown*
5	193760	Ethiopia	Shewa	NU 51642
6	193959	Ethiopia	Shewa	NU 51643
7	194251	Ethiopia	Kefa	NU 51645
8	194252	Ethiopia	Kefa	NU 51646
9	194253	Ethiopia	Kefa	NU 51647
10	194254	Ethiopia	Kefa	NU 51648
11	194255	Ethiopia	Kefa	NU 51649
12	194900	Ethiopia	Gonder	NU 51651
13	194901	Ethiopia	Gonder	*Unknown*
14	194903	Ethiopia	Gonder	NU 51653
15	194904	Ethiopia	Gonder	*Unknown*
16	195552	Ethiopia	Welo	NU 40525
17	195923	Ethiopia	Welo	NU 51657
18	196836	Ethiopia	Welega	*Unknown*
19	197402	Ethiopia	*Unknown*	*Unknown*
20	197403	Ethiopia	*Unknown*	NU 51660
21	199947	Ethiopia	*Unknown*	NU 51661
22	199949	Ethiopia	Gonder	NU 16543
23	199950	Ethiopia	Kefa	NU 51663
24	209023	Puerto Rico	*Unknown*	*Unknown*
25	226545	Ethiopia	Shewa	NU 51664
26	231046	Ethiopia	Shewa	*Unknown*
27	273636	Ethiopia	Harer	NU 51666
28	273640	Ethiopia	Shewa	Gomenzer
29	274283	Ethiopia	*Unknown*	NU 51668
30	280230	Ethiopia	*Unknown*	NU 51669
31	331377	Ethiopia	*Unknown*	*Unknown*
32	331378	Ethiopia	*Unknown*	*Unknown*
33	360879	Sweden	*Unknown*	68-5702-1
34	360880	Sweden	*Unknown*	68-5702-4
35	360881	Sweden	*Unknown*	68-5702-6
36	360882	Sweden	*Unknown*	68-5702-10
37	360883	Sweden	*Unknown*	68-5702-16
38	360885	Sweden	*Unknown*	68-5702-12 PLT 1
39	360886	Sweden	*Unknown*	68-5702-15 PLT 9 S
40	360887	Sweden	*Unknown*	68-5702-16 PLT 5 S
41	390133	Pakistan	*Unknown*	P-1
42	390134	Pakistan	*Unknown*	P-58
43	596535	Spain	Cordoba	BC-815-2
44	596536	Spain	Cordoba	BC-876-2
45	596537	Spain	Cordoba	BC-738-5
46	596538	Spain	Cordoba	BC-834-2
47	596539	Spain	Cordoba	BC-831-2
48	597822	Sweden	*Unknown*	85-0572-69
49	633075	Tanzania	*Unknown*	Swahili
50	633076	Kenya	*Unknown*	WIR 4325
53	633080	Zambia	*Unknown*	BRA 1028/79

filtered vitamins myo-inositol (100 mg/l), thiamine-HCl (10 mg/l), pyridoxine (1 mg/l) and nicotinic acid (1 mg/l) were added to the medium. Seeds were sown at a density of 20-25 seeds per plate, incubated overnight at 15ºC in the dark and then transferred to a culture room at 23ºC under a 16-h photoperiod of 70 µmol m^{-2} sec^{-1} for 72h.

Cotyledons containing petioles of 1-2 mm in length were excised from 4-day-old seedlings and placed at a density of 10-11 cotyledons per plate into 20 x 90 mm Falcon dishes containing 50 ml of regeneration medium. This medium was the same germination medium described above plus 2 mg/l of 6-benzylaminopurine and 500 mg/l of carbenicillin. Explants were cultured in this regeneration medium for three weeks at 23ºC under 16-h photoperiod and then transferred onto fresh regeneration medium and subcultured for one week. Then, they were evaluated for blackening in the petiole, formation of callus or swelling in the base of the petiole and finally for the number of shoots produced via callus phase and via direct regeneration.

2.3. Statistics

Data was analyzed using the SPSS version 11.0 statistical software package. Arcsine√x transformation was carried out on blackening, callus, swelling and no response frequencies before analysis. The General Analysis of Variance and the LSD pairwise comparisons of means were used to determine significant differences.

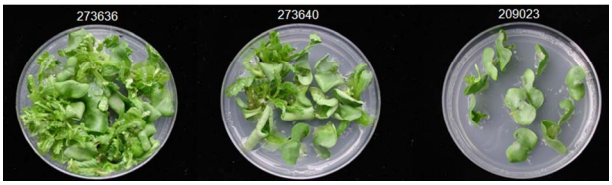
3. Results

Shoot regeneration from cotyledons in the genus *Brassica* can be produced via an indirect callus phase (**Figure 1(a)**). However, not all genotypes produce callus and, in some genotypes, direct shoot regeneration is observed via swelling at the petiole base (**Figure 1(a)**). The occurrence of blackening is thought to result from an interaction between the cut surface of the cotyledon petiole and the medium, and is highly genotype dependent (**Figure 1(a)**). The absence of tissue culture blackening is an important factor for transformation success in *B. oleracea* and *B. napus* [22,24].

In this work we have evaluated the *in vitro* culture response, *i.e.* callus formation, swelling and blackening, and shoot regeneration in 51 genotypes of *B. carinata* and in two DH lines used as controls. **Table 2** shows the results for all genotypes, indicating the number of cultivated explants, the explants with no response to *in vitro* culture, the explants with blackening, and the explants regenerating either via callus or via swelling at the base of the petiole.

Positive response to *in vitro* culture, as determined by callus formation or swelling of explants, was observed in

(a)



(b)

Figure 1. (a) Mode of shoot regeneration: callus formation (left), swelling at the base of the petiole (center) and tissue blackening (right). (b) Variability in number of shoots per explant regenerated via callus in three genotypes of *Brassica carinata*: 273636 (high number of shoots), 273640 (average number of shoots) and 209023 (low number of shoots).

all the genotypes tested (**Table 2**). In genotypes 4, 29, 31 and 1012, all the cultivated explants responded to *in vitro* culture, whereas genotypes 7, 18 and 41 showed the highest percentage of non-responding explants. Although genotypes showed regeneration both via callus and via swelling, most of the genotypes regenerated better via callus phase than via swelling, showing higher percentages of explants regenerating via callus than via swelling (**Table 2**). Only 7 genotypes (7, 8, 9, 10, 12, 14 and 17) showed higher percentage of swelling than callus formation. The maximum percentage of explants producing swelling at the base of the petiole occurred in genotype 9 with 76.9%, while the maximum percentage of callus formation occurred in genotype 29 with 100% of the explants responding via callus formation (**Table 2**). Tissue culture blackening occurred in 2.3% of the 2795 cultivated explants and affected 11 of the 53 genotypes tested. The maximum percentage of blackened explants was 34.6% for genotype 7. This genotype also showed the highest percentage of non-responding explants (57.7%) and all explants regenerated via swelling (**Table 2**).

Figure 2 shows the number of shoots per explant regenerated either via callus phase or via swelling phase (direct regeneration). The mean number of shoots per explant regenerated via callus was 2.9, and the maximum value was observed in genotypes BC71 and 31 with 8.7 shoots per explant each. Six genotypes with more than 6 shoots per explant produced via callus were identified (21, 27, 31, 32, 46 and 71) while twenty-five genotypes produced less than 2 shoots per explant. Figure 1B shows three genotypes with maximum, average and minimum shoot regeneration via callus phase.

Table 2. *In vitro* culture response of cotyledonary explants from a collection of *Brassica carinata* and two DH lines: BC71 (*Brassica carinata*) and AG1012 (*Brassica oleracea*).

Code number	Accession number	Number of explants	No response		Blackening		Callus		Swelling	
			No.	(%)	No.	(%)	No.	(%)	No.	(%)
1	193459	66	2	3.0	0	0	55	83.3	9	13.6
2	193460	47	5	10.6	0	0	36	76.6	6	12.8
3	193467	63	10	15.9	0	0	35	55.6	18	28.6
4	193759	50	0	0	0	0	39	78.0	11	22.0
5	193760	64	3	4.7	12	18.8	61	95.3	0	0
6	193959	46	3	6.5	0	0	38	82.6	5	10.9
7	194251	26	15	57.7	9	34.6	0	0	11	42.3
8	194252	28	4	14.3	5	17.9	11	39.3	13	46.4
9	194253	39	7	17.9	7	17.9	2	5.1	30	76.9
10	194254	49	10	20.4	0	0	13	26.5	26	53.1
11	194255	40	1	2.5	0	0	37	92.5	2	5.0
12	194900	38	4	10.5	0	0	7	18.4	27	71.1
13	194901	52	3	5.8	0	0	46	88.5	3	5.8
14	194903	41	7	17.1	0	0	13	31.7	21	51.2
15	194904	47	15	31.9	1	2.1	22	46.8	10	21.3
16	195552	57	12	21.1	0	0	42	73.7	3	5.3
17	195923	27	5	18.5	0	0	5	18.5	17	63.0
18	196836	45	25	55.6	0	0	20	44.4	0	0
19	197402	50	3	6.0	0	0	44	88.0	3	6.0
20	197403	39	2	5.1	0	0	36	92.3	1	2.6
21	199947	48	6	12.5	0	0	42	87.5	0	0
22	199949	29	8	27.6	0	0	19	65.5	2	6.9
23	199950	38	1	2.6	1	2.6	29	76.3	8	21.1
24	209023	65	11	16.9	0	0	48	73.8	6	9.2
25	226545	29	3	10.3	4	13.8	24	82.8	2	6.9
26	231046	33	5	15.2	0	0	28	84.8	0	0
27	273636	55	2	3.6	0	0	53	96.4	0	0
28	273640	51	5	9.8	0	0	46	90.2	0	0
29	274283	61	0	0	0	0	61	100.0	0	0
30	280230	60	2	3.3	4	6.7	58	96.7	0	0
31	331377	58	0	0	0	0	53	91.4	5	8.6
32	331378	63	5	7.9	0	0	49	77.8	9	14.3
33	360879	56	7	12.5	0	0	47	83.9	2	3.6
34	360880	59	6	10.2	0	0	49	83.1	4	6.8
35	360881	50	12	24.0	0	0	36	72.0	2	4.0
36	360882	65	6	9.2	0	0	55	84.6	4	6.2
37	360883	58	17	29.3	0	0	41	70.7	0	0
38	360885	54	2	3.7	4	7.4	46	85.2	6	11.1
39	360886	63	12	19.0	0	0	50	79.4	1	1.6
40	360887	66	6	9.1	0	0	60	90.9	0	0
41	390133	65	23	35.4	0	0	42	64.6	0	0
42	390134	66	2	3.0	0	0	52	78.8	12	18.2
43	596535	33	5	15.2	0	0	25	75.8	3	9.1
44	596536	39	3	7.7	9	23.1	36	92.3	0	0
45	596537	48	15	31.3	0	0	33	68.8	0	0
46	596538	66	5	7.6	0	0	59	89.4	2	3.0
47	596539	64	8	12.5	0	0	53	82.8	3	4.7
48	597822	76	6	7.9	0	0	63	82.9	7	9.2
49	633075	53	5	9.4	0	0	42	79.2	6	11.3
50	633076	44	4	9.1	0	0	36	81.8	4	9.1
53	633080	95	15	15.8	0	0	74	77.9	6	6.3
71	BC71	44	5	11.4	0	0	39	88.6	0	0
1012	AG1012	127	0	0	3	2.4	100	78.7	27	21.3

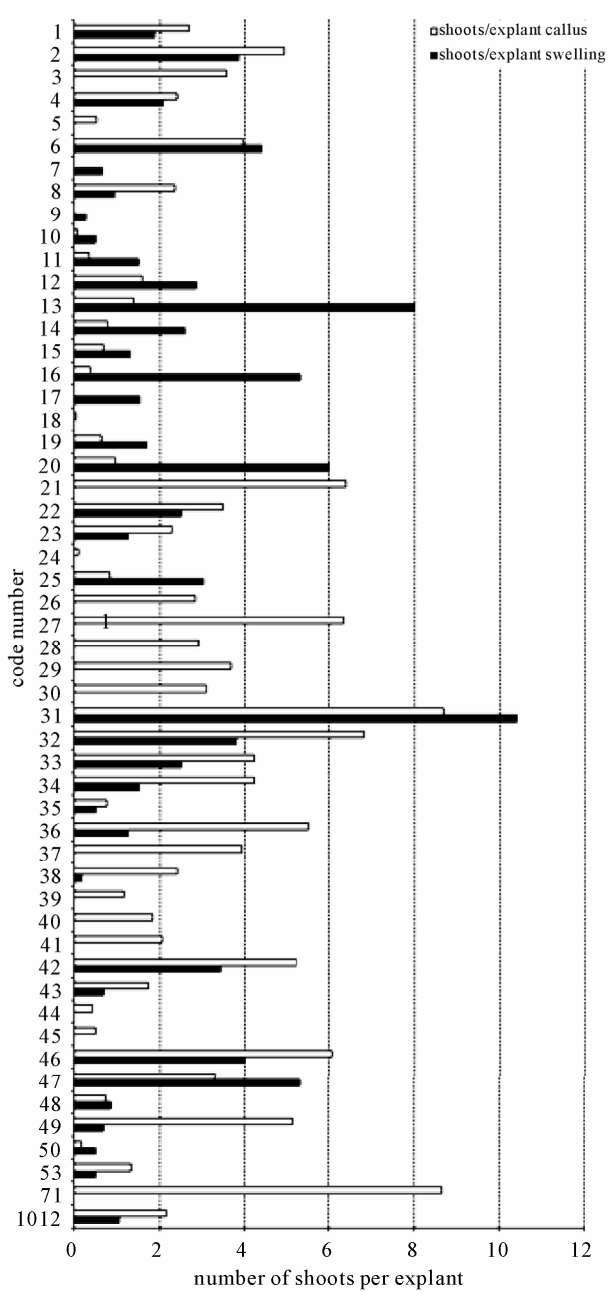


Figure 2 Number of shoots per explant regenerated via callus or swelling from cotyledonary explants collected from 51 lines of *Brassica carinata*. Two DH lines, BC71 (*Brassicca carinata*) and AG1012 (*Brassica oleracea*) were included for comparison.

The mean number of shoots regenerated via swelling was 1.7, and the genotype producing the maximum number of shoots per explant was also genotype 31 with 10.4 shoots per explant. Only three genotypes (20, 13 and 31) produced more than 6 shoots per explant by direct regeneration while thirty-seven genotypes produced less than 2 shoots per explant.

In thirty-two genotypes, shoots were produced both by callus and swelling phase, whereas seventeen genotypes regenerated shoots only by callus phase and in genotypes 7, 9 and 17 all the shoots were regenerated by direct regeneration.

General analysis of variance (**Table 3**) revealed that there were highly significant differences among genotypes for the absence of response to the *in vitro* culture, the mode of response, *i.e.* percentage of callus or percentage of swelling, the occurrence of blackening, and the number of shoots produced either via callus or via direct regeneration.

4. Discussion

Shoot regeneration in *B. carinata* can occur directly from explant tissue as well as indirectly from callus that proliferate from the cut edge of the explants. In many genotypes, direct shoot regeneration has been described as less genotype dependent [25] and regenerants show more genetic stability [26], whereas callus phase is more associated with somaclonal variation. However, direct shoot regeneration has limitations in being used for DNA transfer as totipotent cells are less accessible to *Agrobacterium* during cocultivation [27]. In fact, dedifferentiation of explant cells into callus was necessary for the efficient transformation of *B. carinata* [10]. Therefore, the identification of genotypes with high regeneration potential that regenerate mostly via callus formation can largely influence the recovery of transgenic plants. Sparrow *et al.* [22] found three phenotypic markers highly influencing the transformation efficiency in *B. oleracea*. These are: susceptibility to *A. tumefaciens* [28], shoot regeneration potential and the mode of shoot regenera

Table 3. Analysis of variance for *in vitro* culture response and shoot regeneration in a collection of *Brassica carinata* accessions. Values expressed as a percentage were transformed using the arcsine√x function for the analysis of variance.

Parameter	MS	*df*	F-test
No response (%)	0.1298	52	3.48***
Blackening (%)	0.0476	52	3.14***
Callus (%)	0.1865	52	4.61***
Swelling (%)	0.1617	52	4.90***
Shoots per explant from callus	19.742	51	10.00***
Shoots per explant from swelling	15.596	38	2.16**

Significance probability levels: **1%, ***0.1%. MS; Mean Square, *df*; degree of freedom.

tion. They also found that the absence of blackening tissue associated with callus formation is critical for transformation success. We have applied these criteria to evaluate a collection of 51 genotypes of *B. carinata* for *in vitro* culture and shoot regeneration potential, including the mode of shoot regeneration. We have found a highly positive response to *in vitro* culture, *i.e.* callus formation and swelling, in all the genotypes tested in this study. Other studies have previously confirmed the responsiveness of *B. carinata* seedling explants to tissue culture [29-31]. For both, callus formation and swelling, there were significant differences among genotypes. In our study, the number of genotypes that proliferate via callus formation was much higher than those via swelling. In addition, the percentage of callus formation was higher than swelling in all the genotypes, except in 7 genotypes (7, 8, 9, 10, 12, 14 and 17) in which the percentage of swelling formation was higher.

Callus formation at the base of the petiole has been demonstrated to be an interesting trait in obtaining a great number of shoots per explant. Sparrow *et al.* [22] observed that shoot regeneration via the callus phase resulted in well established and morphologically defined shoots that were easy to isolate and propagate. Results reported here showed that shoot production was very variable among genotypes. This result agreed with studies in *Brassica spp.*, which also reported that shoot production was highly variable among different genotypes, indicating that the regeneration ability is strongly influenced by genotype [15-20]. Zhang *et al.* [18] reported a maximum of 10.7 shoots per explant in a cultivar of Chinese cabbage. In our study the maximum number of shoots per explant was 8.7 and 10.4, regenerated respectively from callus and direct regeneration, both by genotype 31. We have found six genotypes (21, 27, 31, 32, 46 and 71) producing more than 6 shoots per explant via callus formation, although three of them (31, 32 and 46) also produced shoots via swelling. These six genotypes had a percentage of callus formation between 77.8% and 96.4% and no blackening tissue at the base of the petiole was observed. These six genotypes produced an average of 7.1 shoots per explant via callus. This average is 2.4 times higher than the 2.9 shoots of average produced via callus by all genotypes. This high-frequency of regeneration from callus might be of great importance in regeneration of transgenic plants. Mukhopadyay *et al.* [27] reported that the transformation frequency of *B. campestris* is strongly influenced by the mode of shoot regeneration. In addition, Babic *et al.* [10] reported that efficient regeneration from callus was responsible for the high frequency of transformation in *B. carinata*.

The occurrence of tissue blackening resulted from an interaction between the cut surface of the cotyledon petiole and the medium. Although blackening does not prevent the formation of shoots, we have observed a negative correlation between the percentage of blackening and the number of shoots per explant. In addition, genotypes with no blackening produce more shoots than those presenting blackening. Therefore, the absence of tissue blackening influences the successful regeneration of shoots and this could be a critical factor to regenerate transgenic plants.

In conclusion, in this work we have characterized the tissue culture response and the mode of regeneration of a collection of *B. carinata*. The mode of shoot regeneration is important in the selection of genotypes with a high regeneration potential and that regenerate mainly from callus. Six genotypes (21, 27, 31, 32, 46 and 71) have been identified as having a high regeneration potential. They produce the highest number of shoots per explant via callus formation and do not present tissue blackening at the base of the petiole. We are currently incorporating these 6 genotypes in transformation experiments to determine their susceptibility to *A. tumefaciens*. The results of these experiments should allow us to develop a transformation program using genotypes highly susceptible to *A. tumefaciens* and with a high level of regeneration via callus.

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