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Original Research Article

In vitro selection for *Fusarium oxysporum* f. sp. *conglutinans* resistance in *brassica* vegetables

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ABSTRACT

Background: Fusarium wilt is an issue of concern in economically and nutritionally important Brassica vegetable cultivation. Thus, it deserves measures against the adverse production impact caused by Fusarium wilt.
Methods: In this study, development of resistance to *F. oxysporum* f. sp. *conglutinans* in six white head cabbage cultivars, by in vitro chemical mutagenesis and selection, through direct and indirect organogenesis was examined. 6 day and 10 day old hypocotyl, shoot tip and calli, from 6 day old hypocotyl explants, were subjected to chemical mutagenesis treatment (DMSO (4% v/v) + EMS (0.3% v/v) for two hours at 28±2°C) were incubated in MS shoot induction medium (MS+ NAA (0.2 mg/l), BAP (3 mg/l), GA3 (0.01 mg/L) and AgNO3 (0.5 mg/l)). Shoots developed from hypocotyl and shoot tip explants (in the MS shoot induction medium and then in MS + NAA (0.2 mg/l), BAP (3 mg/l), GA3 (0.01 mg/l) for shoot development) and calli following mutagenesis treatment were screened for Fusarium resistance subjecting to 15% and 20% *Fusarium* culture filtrate for 30 and 60 day selection periods for each strength respectively.
Results: Developed plantlets from all six cultivars tested, showed resistance to Fusarium culture filtrate in the in vitro conditions with different survival frequencies ranging between 12.5% to 84.0% from hypocotyl and 0.0% to 86.7% from shoot tip explants among cultivars indicating development of resistance to *Fusarium* by in vitro chemical mutagenesis.
Conclusions: Direct organogenesis, and 10 day old hypocotyl and 6 day old shoot tips are potential explants for successful application of in vitro chemical mutagenesis for Fusarrium resistance development in in cabbage.

Keywords: *Fusarium*, *Brassica*, Cultivar, *In vitro* mutagenesis and selection, Resistance

INTRODUCTION

Brassica oleracea L. var. *capitata* (cabbage) is an economically and nutritionally important vegetable crop grown all over the world. Fusarium wilt is a major and soil borne disease caused by the fungus *Fusarium oxysporum* f. sp. *conglutinans* (FOC) resulting crop damage leading to plant death, affecting production in cabbage cultivation especially in disease susceptible varieties, resulting in sever yield and quality losses.

Cultivation of resistant varieties provides better control of the disease, while chemical and other traditional control methods are ineffective in stemming the disease control after the fungus has become established in the field.[1] Some cabbage cultivars have naturally borne resistance against Fusarium infection and disease development.[1-7] In conventional plant breeding programs these natural inherited resistant traits are used to develop new varieties with a higher resistance capacity, and this is a time consuming exercise requiring many years to achieve desired level of resistance following back crossing. Yet, limited availability of these desired resistance genes in

the germplasm basis causes difficulties with breeding resistance quickly. Expansion of genetic variability in the cultivars by means of modern technologies is time advantageous. In this case, *in vitro* tissue culture techniques and induced mutagenesis provide a great platform and opportunity to broaden the genetic variations in crop improvement. Artificial mutation induction has now emerged, bringing with it faster and greater variability in the crop, resulting in superior plant varieties.[8,9] Van den Bulk has pointed out the importance of *in vitro* tissue culture application to cultivate disease resistance. *In vitro* mutation induction and selection can speed up the breeding program, enhancing the rapid recovery of a desired variation not previously found in the natural gene pool or lost during evolution.[10-11] The acquired resistance through *in vitro* techniques would show longitudinal resistance characteristics and would be hard to break by the emergence of new races and maintained for a longer period. Therefore, *in vitro* mutagenesis and selection provide an efficient, fast and reliable platform for developing new traits in the cabbage resistance breeding program.

The aim of the present study was the development of *Fusarium oxysporum* f. *sp. conglutinans* resistance in *Brassica oleracea* L. var. *capitata* (cabbage) using an *in vitro* culture technique through *in vitro* chemical mutagenesis and selection. The efficiency of resistance development was evaluated in six cultivars of *Brassica oleracea* L. var. *capitata*, three types of explant and two types of *in vitro* regeneration methods (direct organogenesis and indirect organogenesis).

METHODS

Analysis of *in vitro* organogenesis efficiency of selected cabbage cultivars

Seeds of six cultivars of *Brassica oleracea L. var. capitata L.*; Pourovo pozdní (PP), Kiklop F1 (KI), Zeus F1 (ZF), DC 6 (DC), Target F1 (Ta) and Albatros F1 (AL), obtained from the seed company; Moravoseeds CZ a.s. in the Czech Republic, were surface sterilized by 20% Clorox (NaOCl) with 2-3 drops of Tween 20 for twenty minutes followed by a one minute wash with 70% ethanol. Finally, the seeds were washed three times with sterilized distilled water under aseptic conditions. The sterilized seeds were germinated on a seed germination medium containing MS medium (Murashige and Skoog, 1962) supplemented with Sucrose (10 g/l), phytagel (7 g/l) at pH 5.8 and the cultures incubated in light at 25±2ºC.

Three different explants; hypocotyl, shoot tip and cotyledons excised from 6 day and 10 day old *in vitro* derived seedlings were cultured and incubated separately on petri dishes (10 explants per dish and 5 dishes per cultivar) containing MS shoot regeneration medium (15 ml) supplemented with Nicotinic acid (1 mg/l), Thiamine-HCl (10 mg/l), Pyridoxine-HCl (1 mg/l), Myoinositol (100 mg/l), NAA (0.2 mg/l), BAP (3 mg/l), GA_3 (0.01 mg/l), $AgNO_3$ (0.5 mg/l), Sucrose (20 g/l), and phytagel (7 g/l) at pH 5.8 at 25±2ºC in light for direct shoot induction and only hypocotyl and cotyledon explants were cultured and incubated separately on petri dishes (10 explants per dish and 5 dishes per cultivar) with MS callus induction medium (15 ml) supplemented with Nicotinic acid (1 mg/l), Thiamine-HCl (10 mg/l), Pyridoxine-HCl (1 mg/l), Myoinositol (100 mg/l), NAA (0.2 mg/l), BAP (3 mg/l), $AgNO_3$ (0.5 mg/l), Sucrose (20 g/L), and phytagel (7 g/l) at pH 5.8 at 25±2ºC in light for callus induction.

After three weeks in shoot inducing culture (direct shoot induction) they were transferred repeatedly to a fresh identical regeneration medium; the calli derived in the callus induction medium for two to three weeks were transferred to the same shoot induction medium (indirect shoot induction) as was used in direct shoot induction, and incubated for a few weeks with repeated transfers to a fresh medium, till they induced clear multiple shoots approximately 0.5 cm in height.

Shoots derived from both the direct and indirect shoot induction protocols were transferred to the MS shoot development medium supplemented with Nicotinic acid (1mg/L), Thiamin-HCl (10 mg/l), Pyridoxine-HCl (1mg/L), Myoinositol (100 mg/l), NAA (0.2 mg/l), BAP (3 mg/L), GA_3 (0.01 mg/l), Sucrose (20 g/L), and phytagel (7 g/l) at pH 5.8 and incubated for 15 to 30 days at 25±2ºC in light till at least 2 cm in height. After sufficient shoot elongation, these shoots were incubated in MS rooting medium supplemented with IBA (0.2 mg/L), Sucrose (10 g/l), and phytagel (7 g/l) at pH 5 at 25±2ºC in light for few weeks (maximum 8 weeks).

Two biological replicates were carried out to increase the reliability of the resuls.

Development of *Fusarium* resistant cabbage plants

The optimal virulence concentration of fungus culture filtrate was determined by incubating the *in vitro* germinated 10 day old seedlings in solid MS shoot development medium (as described above) supplemented with a series of concentrations (5%, 10%, 15%, 20% and 25% v/v) of fungus culture filtrate prepared as described below. Cultures were incubated for 30 days at 25±2ºC in light (results are not reported here). The fungus culture filtrate was prepared by inoculating a small quantity (2 mm × 2 mm) of isolated *Fusarium oxysporium f.* sp. *conglutinans* strain 1, obtained from the Crop Research Institute in Prague, Czech Republic, into Czapek-Dox Liquid Medium (150 ml) from the dehydrated pre prepared product (SIGMA, 35 g/l) at pH 6.6. The fungus culture was incubated at 28±2°C for 21 days in light, unagitated. The cultured liquid medium was then filtered through a filter paper and centrifuged at 8000 rpm for 20 minutes to precipitate the mycelium and conidia. The pH

of the supernatant was adjusted to 5.8 and the filter sterilized using 0.22 µm pore size membrane.

Plant materials; 6 and 10 day old explants (hypocotyls and shoot-tips) and 7 day old calli derived from 6 day old hypocotyl were subjected to mutagenesis treatment; an aqueous solution with filter sterilized dimethylsulphoxide (DMSO) (4% v/v) and ethyl methanesulfonate (EMS) (0.3% v/v) for 2 hours at 28±2°C. After threefold successive washing with sterile distilled water, the plant materials were cultured either on MS shoot-induction medium for several weeks or on MS callus induction medium for one week.

Following successful recovery after mutagenesis treatment, calli were subjected to resistant screening by incubating them in solid callus induction medium (10 calli per dish) exceptionally containing fungus culture filtrate (15% v/v) for 30 days at 25±2ºC in light. The surviving calli were incubated again for another 60 days in the-same callus induction medium containing (20% v/v) for further selection. Finally, shoots were developed from the selected calli in the same way as described previously. Likewise, for treated calli, the selection process was carried out for regenerated shoots from explants directly following successful mutagenesis treatment and shoot induction. Surviving shoots were rooted and acclimatized. Shoots derived from untreated hypocotyls and untreated calli were subjected to same resistant screening process as controls.

Two biological replicates were carried out in both cases to increase the reliability of results.

The data was subjected to analysis of variance (factorial ANOVA) using the software STATISTICA and all tables presented in the results generated by the same statistical software.

RESULTS

Analysis of in vitro organogenesis efficiency of selected cabbage cultivars

Within the first week in the regeneration medium, pale yellow hypocotyl explants turned greenish and tumescent, while shoot tip explants increased in volume both length and girth, growing the main shoot tip, and cotyledon explants enlarged to approximately three times their initial size. Within 21 days on the regeneration medium, multiple shoot induction was apparent at the distal ends of the hypocotyl explants of all six cultivars and by day 30, clear multiple shoot induction was observed. Shoot induction and the number of shoots per explant produced by each of the three different explant types in each age group through direct organogenesis differed significantly between cultivars, between explant types and between explant age group. Compared to both hypocotyl and shoot tip explants, the cotyledon showed the lowest shoot production in both age groups. Comparatively in general, 6 day old hypocotyl explants showed a lower percentage of shoot induction (maximum 82% in ZF and minimum 43.3% in Ta) than 10 day old hypocotyls (maximum 100% in PP and minimum 22% in DC) (Table 1). However, 6 day old hypocotyl showed a higher production of the transferable number of shoots per explant (maximum 5.2±0.8 in PP and minimum 1.2±0.2 in Ki) after 45 to 60 days in shoot induction medium than the10 day old hypocotyls (maximum 1.9±0.6 in Ki and minimum 0.5±0.2 in DC).

Comparing all six cultivars, ZF, PP and Ta showed better performance with clear multiple shoot induction by day 30 in the shoot induction medium. By the days 45 to 60, there were clear signs of shoot growth from both 6 day and 10 day old hypocotyls of all cultivars. Generally, by day 14-21, shoot tip explants were beginning to induce multiple shoots at the base of the shoot tip and by day 45 evident multiplication of shoots was observed. However, different cultivars showed different responses to multiple shoot induction, in both 6 day and 10 day old shoot tip explants. Cultivars, PP, ZF showed the highest multiple shoot induction from both 6 day and 10 day old shoot tip explants with 86.7% / 92% for PP and 72% / 80% for ZF respectively from the 6 day and 10 day old explants. Both age groups of cotyledon explants in all cultivars showed very low multiple shoot induction and clear development. The DC cultivars didn't show any shoot induction in either the 6 day old nor 10 day old cotyledons.

In indirect organogenesis, both 6 day and 10 day old hypocotyl explants of all six cultivars showed yellowish callus induction within 14 days on the callus induction medium, the percentage ranging from 46.0% to 95.3%. Yet the 6 day old hypocotyl showed better performance with more than 60.0% callus induction in all cultivars ranging from 64.0% to 95.3%. There was no significant difference in callus induction between 6/10 day old hypocotyl explants or between all the cultivar except cultivar Ki. Both 6 and 10 day old hypocotyl explants gave higher callus induction than cotyledon explants, a significantly difference. Overall, in all six cultivars, the calli derived from both the 6 day and 10 day old hypocotyl explants showed significantly similar multiple shoot induction ranging from 42.0% in Ki to 96.0% in PP. Calli derived from 6 day old hypocotyl had a higher shoots development count per explant (maximum 1.9±0.1) than calli derived from 10-day old hypocotyl explant (maximum 0.8±0.1). Interestingly, in all cases 6 day old explants gave a better percentage of shoot induction and better production of shoots per explant in all cultivars and between two types of explants; taking hypocotyl vs cotyledon, hypocotyl explants showed better performance in indirect organogenesis for selected *Brassica* cultivars.

Table 1: Average number of shoots per explant and percentage of total number of shoots developed through direct and indirect organogenesis from different explants excises from 6 day and 10 day old seedlings of six cultivars after 45-60 days of culture initiation.

		Cultivar	*Explant*					
			Hypocotyl		Shoot tip		Cotyledon	
			Explants/calli with shoots (%)	Average no. of shoots/explant, calli (±SD)	Explants with shoots (%)	Average no. of shoots/explant (±SD)	Explants/calli with shoots (%)	Average no. of shoots/explant,calli (±SD)
Direct organogenesis	6-day	ZF	82	2.8±0.5	72	5.9±0.6	30	1.3±0.5
		PP	58.7	5.2±0.8	86.7	3.2±0.4	20	1.3±0.5
		DC	47.3	2.5±0.2	32	1.2±0.1	0	0
		Ki	52.7	1.2±0.2	20	0.6±0.6	5	2.0±1.6
		Ta	43.3	2.0±0.4	8	0	1.7	0.3±0.4
		AL	46.7	3.4±0.5	40	0.7±0.2	1.7	0.3±0.4
	10-day	ZF	98	1.6±0.2	80	3.1±0.5	15	1.3±0.3
		PP	100	1.5±0.4	92	3.3±0.4	30	0.7±0.1
		DC	22	0.5±0.2	24	0.9±0.2	0	0
		Ki	52.7	1.9±0.6	40	1.3±0.7	1.7	0.3±0.5
		Ta	56	0.9±0.1	16	0.5±0.4	0	0
		AL	90	1.1±0.5	1.3	0.3±0.5	0	0
Indirect organogenesis	6-day	ZF	90	1.7±0.2	-	-	40	1.3±0.5
		PP	96	1.9±0.1	-	-	55	1.6±0.8
		DC	52	1.4±0.3	-	-	25	0.7±0.5
		Ki	48	1.2±0.2	-	-	35	0.2±0.2
		Ta	78	1.3±0.2	-	-	30	1.3±0.4
		AL	54	1.2±0.1	-	-	20	0.9±0.3
	10-day	ZF	92	0.8±0.1	-	-	90	1.3±0.3
		PP	94	0.9±0.1	-	-	65	1.3±0.2
		DC	50	0.5±0.1	-	-	0	0
		Ki	42	0.8±0.1	-	-	35	0
		Ta	76	0.7±0.1	-	-	20	0
		AL	70	0.4±0.1	-	-	1.7	0

Development of *Fusarium* resistant cabbage plants

Within the first week in the MS shoot induction medium, following the mutagenesis treatment, mortality of some explants were observed in all cultivars regardless types or the age group of explant used. Within 21 days, the surviving explants started to produce multiple shoots at the distal cut ends of the hypocotyl and base of the shoot tip, with different capacity levels between cultivars and explant age group (Figure 3).

There was no significant difference in shoot induction between types of explant, but there was a significant difference between explant ages. As observed (Figure 1B), 10 day old explants showed a higher percentage of shoot induction (the minimum ranging from 8.0% to 30.0% and the maximum ranging from 60.0% to 62.0%) than 6 day old explants (the minimum ranging from 4.0% to 4.7% and the maximum ranging from 40.0% to 38.0%). Among the cultivars, there was a significant difference of shoot induction between the DC, Ki and Ta cultivars after mutagenesis treatment and there was no significant difference between PP, ZF and AL cultivars.

After mutagenesis treatment, there was no significant difference in the number of shoots induced per explant between types of explant, but there was a significant difference between explant ages. Also between cultivars PP, ZF and DC, there was a significant difference in the number of shoots per explant produced. Cultivar PP showed the maximum production of number of shoots per explant in both age groups and two types of explants. The percentage of the number of surviving shoots after both selection cycles (15% v/v for 30 days and 20% v/v for 60 days) was not significantly different within either factor; the type of explant or the age of the explant, but there was a significant difference between some cultivars and between cultivar types and explant ages. Despite there being no statistically significant difference, the observed data showed some differences between each of the cultivar factors; PP, ZF, Ki, AL and Ta showed an increased resistance development rate using 6 day old shoot tip explant (ranging from 36.7 to 86.7%) than hypocotyl explant (ranging from 16.7 to 28.3%) while cultivar DC showed a reduction of resistant mutation development in both age groups (from 66.7% to 16.7% in 6 day old explant and from 46.7% to 0.0% in the10 day

old group). Except cultivars ZF and AL, all other cultivars showed low resistance mutation induction from the 10 day old hypocotyl explant to shoot tip explants (Figure 1C and D).

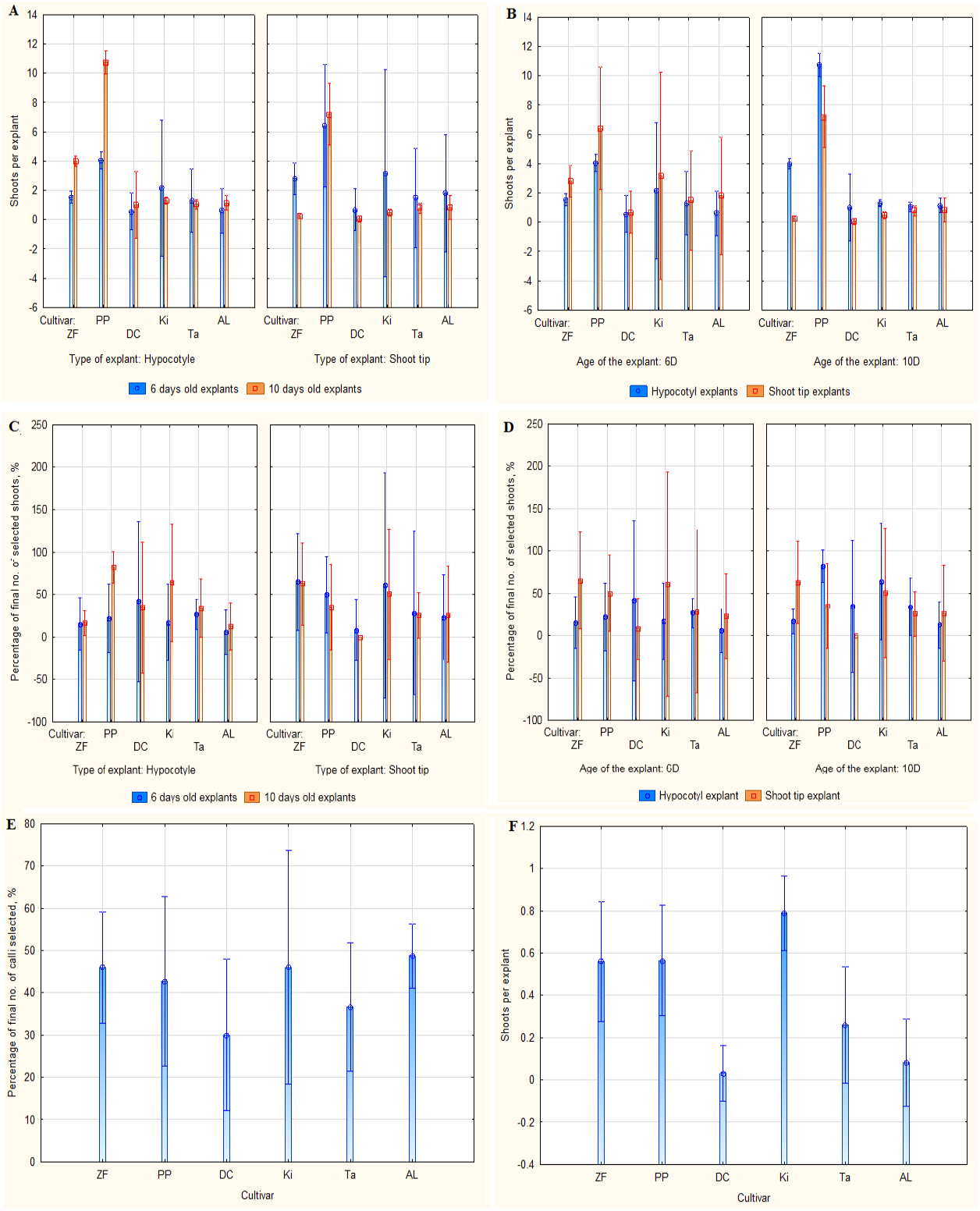

Figure 1: Shoot induction and selection through direct and indirect organogenesis after mutagenesis treatment. (A & B) Number of shoots developed per explant based on explant age and type, (C & D) Percentage of shoots selected showing resistance to culture filtrate after mutagenesis treatment, (E) Percentage of calli selected showing resistance to culture filtrate after mutagenesis treatment, (F) Number of shoots developed per callus after selection.

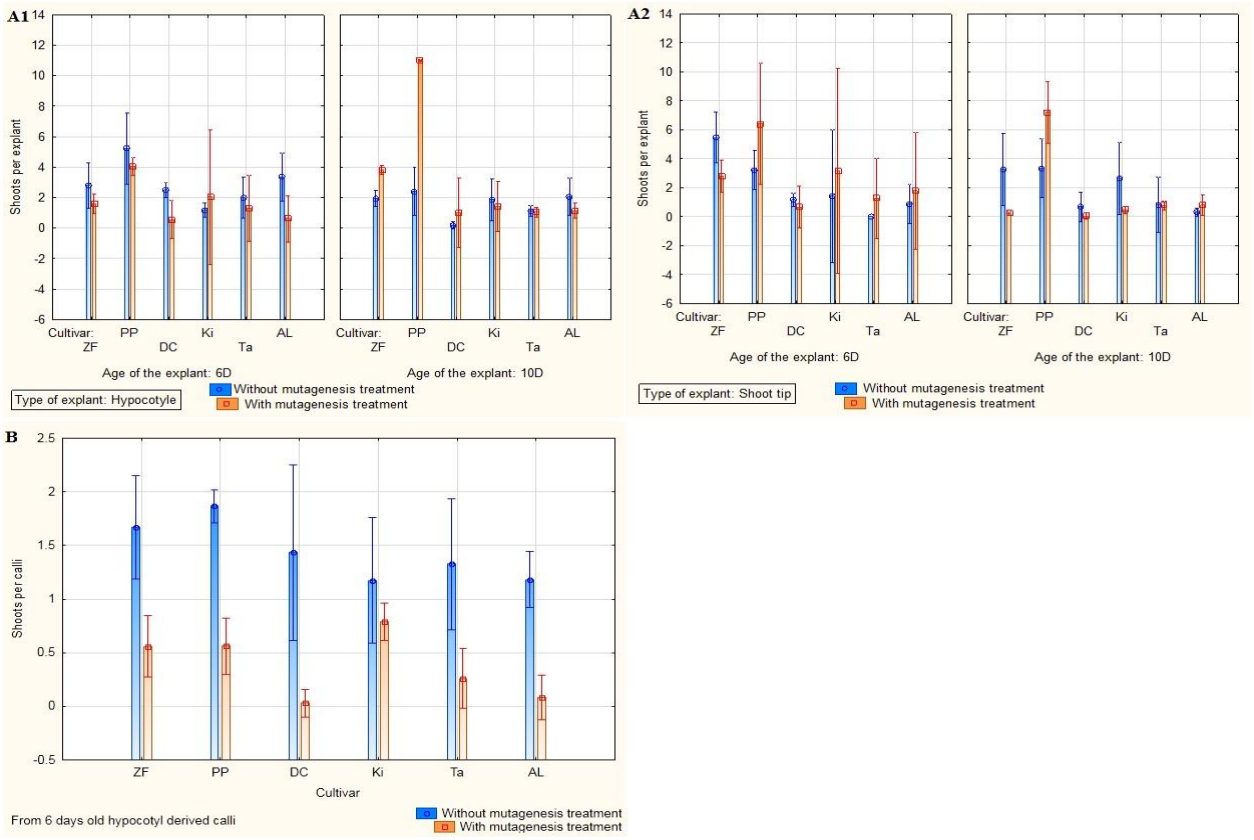

Figure 2: Comparison of in vitro organogenesis between the without mutagenesis and after mutagenesis treatment and selection. (A1 & A2) The number of shoots developed per explant based on explant age and type through direct organogenesis, (B) Number of shoots developed per callus.

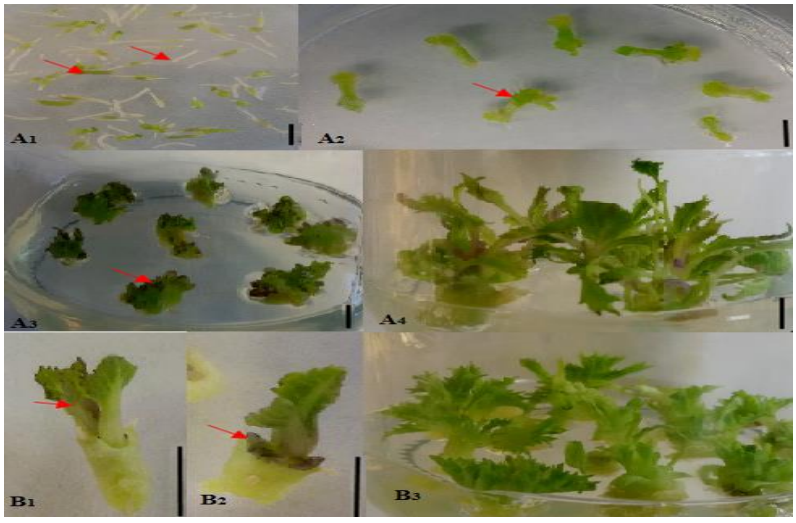

Figure 3: Direct shoot induction after mutagenesis treatment from hypocotyl and shoot-tip explants excised from 6 day old seedlings, in MS shoot induction medium. Bar=10 mm. (A) Hypocotyl explant; A1: Green surviving explants and dead explants after 7 days in MS shoot induction medium. A2: Swelling and callus like structure formation after 14 days. A3: Clear multiple shoot induction after 30 days- A4. Well grown shoots after 45 days. (B) Shoot-tip explants; B1: Growth of main shoot and increased size of explant after 7 days. B2: Multiple shoot induction at the base of the shoot tip after 14 days. B3: Clear multiple shoot development after 21-30 days.

After mutagenesis treatment and *in vitro* selection, surviving yellow-green calli were started shoot induction in the first week in a shoot induction medium. Nevertheless, most of the remained calli after selection stress showed dormant behavior in the shoot induction medium and remained green and hard compact calli without any shoot induction even after 60 days in the shoot induction medium. This dormancy was greatly observed in cultivar DC with zero shoots per callus. Generally, the average number of shoots per callus was very low (Figure 1E and F) in all cultivars after mutagenesis and selection. Interestingly, as also seen in direct organogenesis, there was a significunt difference of induced number of shoots per callus between shoot induction, without subjecting to mutagenesis treatment, and after mutagenesis tratment. Shoot induction was reduced after mutagenesis.

There was a significant difference in shoot induction from each type of explant among cultivars not subjected to mutagenesis treatment and after mutagenesis and selection (Figure 2). But there was no significant difference in shoot induction between the without and with mutagenesis treatment, cases, between the age groups of the explant and between hypocotyl and shoot tip explants. It was markedly observed in cultivar PP, that after mutagenesis it showed a more numerous multiple shoot induction. Yet the callus reduced shoot induction significantly after mutagenesis treatment (Figure 2B).

DISCUSSION

In the *in vitro* tissue culture process, having a reliable and efficient culture protocol is a critical aspect of success. Though there are various explants that can be used as the starting material in plant tissue culture, all such tissues are not suited for all *in vitro* organogenesis techniques and for all plant species. The regeneration efficiency dramatically depends on the explant, the genotype variant, the age of the explant due to the physiological status.[12-14] In this study, three types of explants, hypocotyl, shoot tip and cotyledon, in two age groups, 6 day and 10 day, taken from seedlings, were examined for the regeneration capacity of selected cultivars of *B. oleracea* var. *capitata*. As previous studies have reported, significant differences of regeneration between different explants, ages and cultivars were observed, in both the direct and indirect organogenesis pathways. In direct and indirect organogenesis analysis, the cotyledon explant showed the lowest shoot production (ranging from 0 to 30% shoot induction and 0 to 2.0±1.6 shoots per explant in direct organogenesis and 0 to 90% of callus induction with 0 to 1.6±0.8 shoots per callus in indirect organogenesis) in both the age groups. Gerszbery et al also observed the similarly very low percentage of organogenesis from cotyledon explants of some cultivars of *B. oleracea* var. *capitata*. Yet Qamar et al observed 100% callus induction from hypocotyls of cabbage cultured in a medium supplemented with 2,4-D (2.0 mg/L) and BAP (0.25 to 1.0 mg/L) but a considerably higher dosage of cytokinin was essential for shoot induction from the callus to break the strong auxin effect of 2,4-D.[14,15] They achieved 100% of shoot induction by a combination of BAP (4 mg/L), Zeatin (4 mg/L) and IAA (5 mg/L). Munshi et al also observed the maximum percentage of callus induction from cotyledon explants on 2,4-D (1.0 mg/L) and NAA (0.5 mg/L), and shoot induction was only observed from the calli derived from cotyledon explants.[17]

In direct organogenesis, each cultivar showed different regeneration capacities for each explant type. PP and ZF cultivars showed a better regeneration response in all three explants and all ages, compared to the other four cultivars tested. As could be observed and analyzed from the data, 6 day old hypocotyl explants showed significantly better responses under the tissue culture protocols used, with some exceptions between cultivars. In indirect organogenesis, hypocotyl explants also showed better response for callus induction (46.0 to 95.3% callus induction) with a significant difference in shoots per explant between different cultivars. These results agree with the observations of previously reported studies on *Brassica in vitro* regeneration.[15,17-19]

In this study, we observed a low survival of explants and a reduction of regeneration capacity through indirect organogenesis after treatment, showing dormancy in most of the calli. But through direct organogenesis, the observed results were varied and interesting between cultivars, explants and their ages. The dosage of the mutagen treatment to achieve a desirable mutation effect on the target genome is important, as a higher dosage will cause high mortality of the plant materials. In addition to the higher mortality of plant materials, increased mutagen strength also reduces the regeneration capacity of the plant materials. Yun et al observed, zero percent regeneration from cotyledon explants of *B. rape* after EMS (1% v/v) treatment for 25 hours and after treating with NaN_3 (1000 µM) for 25 hours.[20] As previous studies have noted, reduced shoot production was also observed here, especially from the 6 day old hypocotyl explant in each cultivar, except cultivar Ki, which showed a slight (not significant) increase in shoot induction after mutagenesis treatment. Interestingly, increased shoot production was observed from explants of cultivar PP (10 day old hypocotyl, and both 6 and 10 day old shoot tip explants) following the treatment. In general, 6 day old shoot tip explants showed increased shoot induction in each cultivar, except cultivars ZF and DC. This is an interesting observation, as no previous reported studies on *Brassica in vitro* mutagenesis have studied use of shoot tip explant. Cell free culture filtrate of *Fusarium oxysporium* has been successfully applied for the *in vitro* selection of many species so far; *Musa spp*, *Linum*, turmeric, melon.[21-24] In this study, *Fusarium* culture

filtrate was also successfully applied, achieving efficient selection responses.

As many other study groups have observed, chlorosis and necrosis of cotyledon explants leading to the death of cotyledons during culture was observed in this study.[15,25,26] Accumulation of phenolic compounds and their oxidation products could be one reason for this, as *Brassica* species are rich sources of phenolic compounds. Accumulation of ethylene produced from the *in vitro* growing of plant tissues due to wounding stress can be another reason for this chlorosis and necrosis. Accumulation of ethylene can also cause poor regeneration capacity or abnormal growth of shoots.[27-29] Many study groups observed enhanced shoot regeneration in the *in vitro* cultures of *Brassica* species using ethylene inhibitors.[27,30,31] $AgNO_3$ not only acts as an ethylene inhibitor but also enhances the regeneration efficiency of *in vitro Brassica* cultures.[25,32-35] Bhalla and Weerd have proposed using porous surgical tape (3M micropore) as the sealing material of culture plants, rather than non porous plastic (Nescofilm or parafilm) to increase aeration and reduce the accumulation of ethylene and they also noted a reduction of condensation in the cultures, which was another problem also observed in the present study.[36]

As all reported previous studies emphasized the genotype dependency on *in vitro* organogenesis of *Brassica oleracea*, this study likewise clearly observed a similar response.[12,14-16,19,36-39] After successful mutation induction and selection, we need to evaluate the resistance capacity of developed resistant plants to a live pathogen in both greenhouse and field conditions, in order to confirm the stability of the mutational resistance developed in the plant. Identification of induced mutational resistance gene or genes through *in vitro* mutation induction would be of benefit for future studies. Furthermore, we need to check the vegetative growth and phenotypic characteristics and head production, to validate the influence of mutagenesis treatment on these other characteristics in addition to inducing Fusarium resistance. Such analysis was outside the scope of the present study.

CONCLUSION

This study provides a foundation for the possible application of *in vitro* chemical mutagenesis and *in vitro* selection by culture filtrate toward developing induced novel Fusarium resistance in *Brassica oleracea* var. *capitata* plants, for the future application of this technique as a biotechnological approach to advancing Fusarium resistant cabbage cultivars.

In conclusion, the study results demonstrated direct organogenesis and use of hypocotyl (10 day old) and shoot tip (6 day old) explants as potential explants for *in vitro* mutagenesis of *B.oleracea* var. *capitata* with variations among six cultivars tested. Further studies are needed for optimization of the suitable *in vitro* culture protocol for higher regeneration efficiency in each genotype, to optimize the mutagenesis treatment to achieve a desirable degree of mutation efficiency, with a greater survival percentage, to optimize the of acclimatization protocol and screening of *in vitro* derived resistant plantlets with live pathogen to be promised the resistant for maximizing *in vitro* mutagenesis success toward developing Fusarium resistance in *Brassica.*

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