PLANT REGENERATION OF KAMCHATIC PLANTAIN (*PLANTAGO CAMTSCHATICA* LINK) THROUGH CALLUS INDUCTION

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ABSTRACT

In vitro seedling offspring of Plantago camtschatica Link was investigated regarding induction of somatic embryogenesis in petiole/leaf explants from shoot tissue and shoot proliferation. The aim of study was to investigate the medium supplemented with suitable concentration of plant growth regulators in order to induce somatic embryogenesis, plant regeneration and shoot multiplication. The results showed that: (i) Petiole/young leaf of immature stem induced the highest ratio of calli induction and compact calli formation on MS medium supplemented with 1 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BA; (ii) From created calli, somatic embryogenesis could be induced on MS medium supplemented with 1 mgL^{-1} TDZ or 1 mgL^{-1} TDZ and 0.5 mgL^{-1} NAA; (iii) MS medium supplemented with 5-7 mgL^{-1} BA shown the most effective on shoot development stage; (iv) Rooting of shoot was the best on 1/2 solid MS medium with activated charcoal (2 gL^{-1}), and 0.5-4 mgL^{-1} NAA; and (v) acclimatization of micropropagated plants could be planted in plastic pots containing a mixture of decayed straw : rice husk ashes, (1:1, v/v), sand : soil (1:1, v/v) or soil, showed a high survival rate and most seedlings grew normally.

KEYWORDS: *Plantago camtschatica Link, plant growth regulators, in vitro culture, organogenesis, plant regeneration, acclimatization.*

ABBREVIATIONS

BA: Benzyl adenine
2,4-D: 2,4-Dichlorophenoxyacetic acid
NAA: α-Naphthaleneacetic acid
TDZ: Thidiazuron
MS: Murashige and Skoog
RCBD: Randomized Complete Bock Design

INTRODUCTION

Plantago is a polymorphic genus of Plantaginaceae family consisted of more than 260 species worldwide distributed. Kamchatic plantain- *P. camtschatica* Link, synonym *P. depressa* Wild. subsp. *camtschatica* (Cham. ex Link) Pilg., a perennial medicinal plant belonging to this family [8, 12] contains many biologically active compounds such as iridoid glycosides, polysaccharides,

flavonoids, caffeic acid derivatives and terpenoids. This plant is mentioned as an old medicinal plant that has been used more than a millennium ago for wound healing remedy and in the treatment of a number of diseases which include diseases related to the skin, respiratory organs, digestive organs, reproduction the circulation, anti-cancer, pain relief and against infections [15, 18 & 25]. Recently, this variety has attracted much attention and became economically important [2 & 13].

Plantain propagation through conventional and tissue culture methods were successfully achieved for several other species: *P. asiatica* L., *P. lanceolata* L., *P. major* L., *P. maritima* L., *P. media* L. and *P. ovata* Forssk [9, 16, 17 & 23]. Therefore, our objective of this study was to investigate the effective media for inducing somatic embryogenesis, shoot regeneration and multiplication stages of *P. camtschatica* plant, in order to apply for and improve the plantain production and application, as well as contributing to the development of broadleaf plantain processing industry that its products supplied for both domestic and overseas consumptions.

MATERIALS AND METHODS

Plant materials

The matured seeds of *P. camtschatica* were surface-sterilized by rinsing with 70% ethanol for 2 min and rinsed 3 times in sterile distilled water. Then the seeds were sterilized in 2% (v/v) NaOCl and 0.03% (v/v) Tween 20 for 15 minutes, rinsed 3 times in sterile distilled water. And then the seeds were rinsed by 0.1% (w/v) $HgCl_2$ solution for 2 minutes and finally rinsed 6 times in sterile distilled water.

Media and culture condition

MS basal medium including vitamins, Sigma-Aldrich- USA, [19], plant growth regulators (BA, TDZ, 2,4-D and NAA) were added (depending on *in vitro* cultured stages), and the pH of the media were adjusted to 5.7-5.8 using NaOH/HCl solution. The sterilized seeds were aseptically germinated in jars containing 30 gL^{-1} agar-solidified MS basal medium that free plant growth regulators at growth chamber with temperature of 25±1°C. The culture was placed in the dark and transferred into the light after germinating in 80-90% humidity and light intensity 3000 lux.

Effect of various hormone concentration and combinations on callus induction

The experiment was arranged in a Randomized Complete Bock Design (RCBD) with 16 treatments, 5 replications, and 5 explants per replication for each type of sample. Those treatments were distributed in various combination of 2,4-D concentration (0, 0.1, 0.5 and 1 mgL^{-1}) and BA concentration (0, 0.5, 1 and 2 mgL^{-1}).

Shoot regeneration and multiple shoots

Using the callus clusters selected from the best treatment of experiment above. The experiment was arranged in RCBD with 12 treatments, 5 replications, and 5 explants per each. All treatments were arranged in various combination of NAA concentration (0, 0.5 &1 $mg.L^{-1}$) and TDZ concentration (0, 0.2, 0.5 & 1 mgL^{-1}).

Shoot tips with 3-4 leaves were cultured. The experiment was arranged in a RCBD with 5 replications per treatment, jar (3 shoots, 1.5 - 2 cm height) per replication. Those treatments were distributed in various BA concentrations (0, 1, 2, 3, 5 and 7 mgL^{-1}).

Root induction

The experiment was arranged in a RCBD with 10 treatments, 5 replications, jar (3 shoots, 1.5 - 2 cm height) per replication. Shoot tips with 3-4 leaves were cultured. The treatments were arranged in 2 kinds of MS medium (half and full strength) which were supplemented with activated charcoal 2 gL^{-1} and NAA concentration (0, 0.5, 1, 2 and 4 mgL^{-1}).

Acclimatization

The experiment was arranged in a completely randomized design with 4 treatments, 5 replications and 15 plantlets per replication. The treatments were distributed in 4 substrates including sand, soil, sand : soil (1:1, v/v) and decayed straw : rice husk ashes (1:1, v/v)

Statistical analysis

All the data were analyzed by using the Statistical Package for Social Sciences (Version 16.0 for windows, SPSS Inc.) to perform ANOVA and DUNCAN tests ($P \leq 0.05$ or 0.01).

RESULTS AND DISCUSSION

Effect of various hormone concentration and combinations on callus induction

Plant growth regulators are important in plant tissue culture since they play vital roles in stem elongation, tropism, and apical dominance. Those growth regulators are required in combination in the media as it is always the manipulation and variation of auxins and cytokinins levels that can successfully change the growth behavior of plant cultures [6]. Proportion of auxins to cytokinins determines the type and extent of organogenesis in plant cell cultures [21]. Thus, callus formation was observed in 4 weeks after inoculation from the young leaf and petiole explants on modified MS medium containing different concentration of 2,4- D (0, 0.1, 0.5, 1 mgL^{-1}) and BA (0, 0.5, 1, 2 mgL^{-1}). For the effect hormone combination, maximum callus induction was observed at 2,4-D 1 mgL^{-1} and BA 0.5 mgL^{-1} (96.67%, Table 1), minimum callus induction (6.67%) was observed at 0.1 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BA, and 0.1 mgL^{-1} 2,4-D with 1 mgL^{-1} BA. And no callus induction was observed on any of the concentrations BA (0-2 mgL^{-1}) and concentrations of 0.1 mgL^{-1} 2,4-D. The difference is significant at 0.01 level. However, there are not significant at the effect of types of samples, as well as the interaction between sample type and hormone combination on percentage of callus induction of kamchatic plantain at 4 weeks after culture (Table 1).

Effect of various hormone concentration and combinations on shoot organogenesis

The most effective variables in plant tissue culture media are growth regulators, especially auxins and cytokinins. Thus, among the different concentration and combination of TDZ and NAA for shoot organogenesis of kamchatic plantain (Figure 1), the highest shoot regenerating capacities (96 and 92%) were observed on MS supplemented with 1 mgL^{-1} TDZ or 1 mgL^{-1} TDZ with 0.5 mgL^{-1} NAA combination, respectively. The minimum (4%) shoot regeneration was recorded in 1 mgL^{-1} TDZ plus 1 mgL^{-1} NAA combination, significant at 0.01 level. Whereas, there are not shoot regeneration in any of concentration of 0-0.5 mgL^{-1}TDZ and 0-1 mgL^{-1} NAA, except to the combination of 0.5 mgL^{-1} TDZ and 1 mgL^{-} NAA. Moreover, the shoot organogenesis was not formed on the MS media containing 0.5 or 1 mgL^{-1} NAA, 0.2 mgL^{-1} TDZ, 0.2 mgL^{-1} TDZ combined with 0.5 mgL^{-1} NAA or 1 mgL^{-1} NAA, and 0.5 mgL^{-1} TDZ (data unshown).

Table 1

Effect of various hormone concentration and combinations on percentage of callus induction of *Plantago camtschatica* at 4 weeks after culture

Hormone combination (mgL^{-1})	Sample types		Callus induction, %
	Young leaf	Petiole	
Control (hormone free MS)	0.0	0.0	0.0 e
0.5 BA	0.0	0.0	0.0 e
1 BA	0.0	0.0	0.0 e
2 BA	0.0	0.0	0.0 e
0.1 2,4-D	0.0	0.0	0.0 e
0.1 2,4-D + 0.5 BA	6.7	6.7	6.67 e
0.1 2,4-D + 1 BA	6.7	6.7	6.67 e
0.1 2,4-D + 2 BA	8.3	13.3	11.1 e
0.5 2,4-D	60.0	53.3	56.7 bc
0.5 2,4-D + BA 0.5	26.7	46.7	36.7 d
0.5 2,4-D + BA 1	26.7	73.3	50.0 bcd
0.5 2,4-D + BA 2	33.3	46.7	40.0 cd
1 2,4-D	60.0	66.7	63.3 b
1 2,4-D + 0.5 BA	100.0	93.3	96.7 a
1 2,4-D + 1 BA	66.7	60.0	63.3 b
1 2,4-D + 2 BA	53.3	40.0	46.7 bcd
Callus induction (%) on sample types (B)	28.0	31.7	
P_A	**		
P_B	ns		
P_{A*B}	ns		

Means of five replicates (Petri dishes) with 10 explants each. Means with same letters indicate no significant difference by Duncan's multiple-range test; **: significant at 0.01 level; ns: not significant.

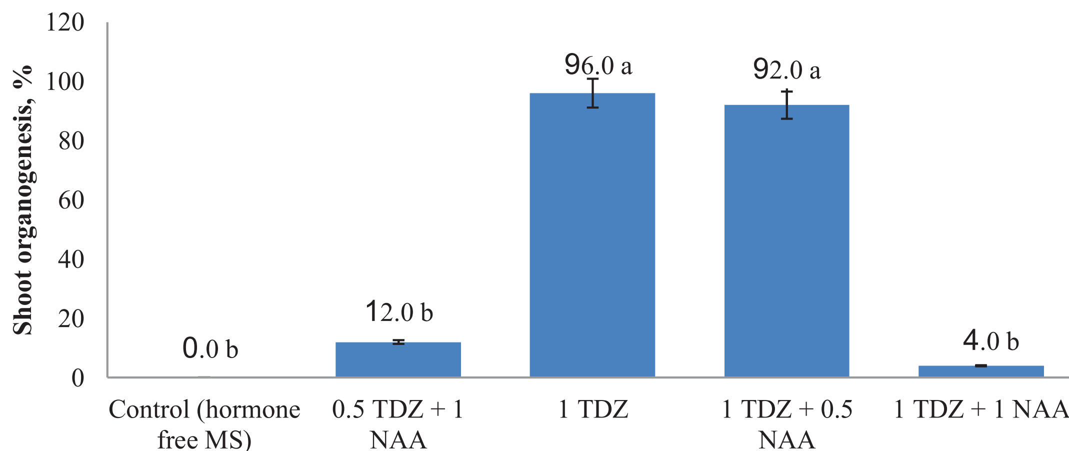

Figure 1. Shoot regeneration from cotyledon and young leaf of *P. camtschtica* on the MS media containing different plant growth regulators after 4 weeks cultured

Means of 5 replicates with 10 explants per each Petri dish. Means with same letters indicate no significant difference by Duncan's multiple-range test; **: significant at 0.01 level.

Effect of BA concentration on *in vitro* shoot multiplication of kamchatic plantain

Apart from the influence of genotypes, shoot proliferation rate and elongation are influenced by cytokinin types and their concentration. Adenine-based cytokinins are commonly used in a large of species for in-vitro propagation, and BA or BAP is the most commonly preferred cytokinin [24]. The concentration of exogenous cytokinin appears to be the main factor affecting multiplication. The findings of this study demonstrated the effects of cytokoinins on shoots formation and multiplication. In this experiment the use of BA alone had significant effect on shoot proliferation (Table 2). Shoot proliferation is important component of quality for micropropagation. It was observed that the response to a range of concentrations of BA was statistically different (P = 0.01) with respect to number of shoots per explants during 8 weeks of culture. New shoots formed on the MS medium with 5 and 7 mgL^{-1} BA, were the highest (2.8, 3.6, 3.7, 3.7 shoots and 3.3, 3.5, 3.8, 4.9 shoots after 2, 4, 6 and 8 weeks cultured, respectively). Those shoots were healthy (Fig.2 C&F) and were ready to root. The shoots were multiplied on a same medium for over 6 months. [20] mentioned that the initial response of explants to shoot formation due to addition of cytokinin is mediated by an increase in the cytosolic calcium concentration which is promoted by its high uptake from the media. This affects cytoskeleton and regulates exocytosis [11]. [22] indicated that for multiplication of propagules, a medium containing a range of concentration 0.1 - 20 mg/ of BA is added to the media. Generally, this study indicates that increasing concentration of BA for this particular variety enhanced the buds formations. Addition of 5-7 mgL^{-1} to the growth media showed best results compared with all other treatments (Table 2). This seems to be the optimal concentration for this variety.

Table 2

Effect of various BA concentrations on number of *in vitro* proliferated shoot of *Plantago camtschatica*

BA concentration (mgL^{-1})	Periods of inoculation (weeks after cultured)			
	2	4	6	8
0 (Control, MS free BA)	1.0 d	1.1 c	1.1 c	1.3 d
1	1.5 cd	1.7 bc	1.8 bc	1.9 cd
3	2.0 bc	2.1 b	2.1 b	2.3 bc
5	2.8 ab	3.6 a	3.7 a	3.7 ab
7	3.3 a	3.5 a	3.8 a	4.9 a
P	**	**	**	**

Means of five replicates (jars) with 5 explants each. Means with same letters indicate no significant difference by Duncan's multiple-range test; **: significant at 0.01 level.

Effect of NAA concentration on root induction of kamchatic plantain

The mineral composition of nutrient solutions should be adapted to each particular situation. Indeed, full strength of salts in media proved good for several species, but in some species the reduction of salts level to ½ or ¼ the full concentration gave better results in *in vitro* growth. Among different concentration and combination of NAA and MS minerals on the *in vitro* root induction of plantain were shown in Tables 3. The results showed positively that the best number of root (13.7) was observed on 1/2 MS medium, better than full strength MS, significantly different at 0.01 level. Thus optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirements. Moreover, tissues from different parts of plants may also have different requirements for satisfactory growth [10 & 19]. In *in vitro* rooting stage, plantlet quality may be effectively improved by reducing of macromineral requirements especially lowering nitrogen concentration [7]. On the other hands, NAA concentration was significant for the response of root number. The best number of root was on medium with 0.5-4 mgL^{-1} NAA (Table 3).

Table 3

Effect of different NAA concentrations and MS macromineral levels on number of root induction of *Plantago camtschatica* at 4 weeks after culture

NAA concentration (mgL^{-1})	MS macromineral levels		Number of root
	Full strength	Half strength	
0	4.2	9.5	6.9 b
0.5	12.3	13.8	13.0 a
1	12.3	16.1	14.2 a
2	13.4	14.7	14.1 a
4	12.1	14.5	13.3 a
Number of root on media types (B)	10.9 b	13.7 a	
P_A	**		
P_B	**		
P_{A*B}	ns		

Means of five replicates (jars) with 5 explants each. Means with same letters indicate no significant difference by Duncan's multiple-range test; **: significant at 0.01 level; ns: not significant.

Effect of different substrates on acclimatization of micropropagated kamchatic plantain

A substantial number of micropropagated plants do not survive transfer from *in vitro* conditions to greenhouse or field environment. The greenhouse and field have substantially lower relative humidity, higher light level and septic environment that are stressful to micropropagated plants compared to in vitro conditions. The benefit of any micropropagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found ex vitro. Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil.

An intermediate hardening stage or acclimatization is therefore necessitated to improve survival rates of such *in vitro* developed plants. During this acclimatization all the factors including air humidity, temperature, irradiance, CO_2 concentration and air flow rates need to be controlled so as to prepare the plant for the *ex vitro* conditions [4, 5 & 14]. Thus the ultimate success of micropropagation on a commercial scale depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates. [3] mentioned that supporting material has a crucial role and could influence on survival percentage, growth and development in acclimatization stage.

In the study, the plantlets with well developed roots were separated from the culture vials without damaging the roots. The media adhering to the roots were washed in running tap water. After washing the roots, all plantlets were transferred to pots with substrates in a plastic tray, covered with lids/plastic bags and kept under the growth room condition for a period of 2 weeks. Then lids/plastic bags were taken off and plants were left to grow in the net house. The results revealed that the survival rate and new number of leaf of shoots was highest with all substrates (93.3-100%), except to sand (86.7%), significant difference at 0.05 level (Table 5). In the net house condition high percentages of plantlets were successfully transferred into potted substrates and they developed into normal plants in the greenhouse with high survival after 4

week culture. Phenotypic variability was not observed in plants (Fig.2H).

Tissue culture techniques have proven to be useful in propagating medicinal plants in controlled conditions. Up to date, *in vitro* culture of *P. camtschatica*. was only initiated by [1] that induced the regeneration of plantlets of this species through indirect organogenesis, and we have reported somatic organogenesis and embryogenesis, micropropagation through the culture of shoot-tips, the ultrastructure of different calli of this species. *In vitro* culture of *P. camtschatica* Link. can support plant material for phytochemical analysis. Thus, regenerated shoots of this plant may contain such crucial important biosubstances as iridoid and other glycosides.

Table 5

Effect of different substrates on the growth of micropropagated *P. camtschatica* after 4 weeks of acclimation in the net house condition

Substrates	Survival (%)	No. of new leaves
Sand	86.7 b	1.9
Soil	93.3 ab	2.1
Sand : Soil (1:1, v/v)	100 a	2.3
Decayed straw : rice husk ashes (1:1, v/v)	100 a	2.5
P	*	ns

Means of five replicates with 15 plantlets each; *: significant at 0.05 level; ns: not significant.

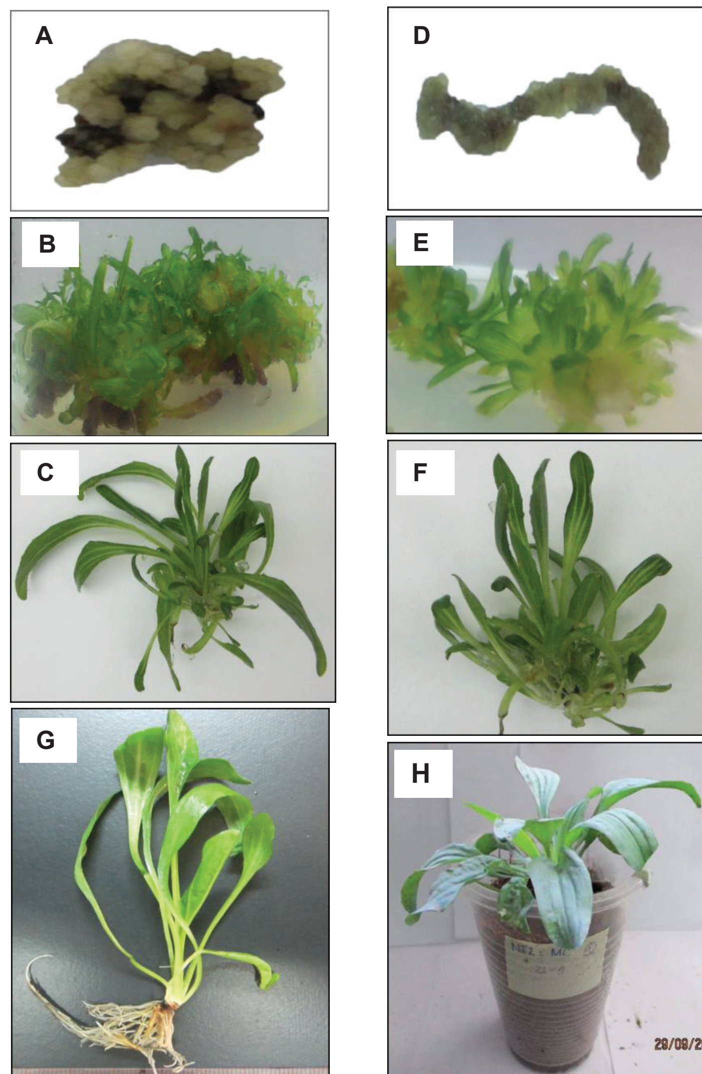

Figure 2. Regeneration stages of *Plantago camtschatica*. Callus induction, shoot regeneration, and multiplication from young leaf (A, B, C) and petiole explants (D, E, F), respectively. G: Rooted shoot after 4 weeks of culture on ½ MS medium supplemented with 0.5-4 mgL^{-1} NAA. H. Micraprapagated *Plantago camtschatica* Link plant after 4 weeks of pot growth.

CONCLUSION

In vitro cultures allow effective micropropagation in particular of *P. camtschatica* with presents a potentially economical item for near future. The result above is efficient enough to be used for mass production of healthy plants in short time.

Petiole or young leaf of immature stem induced the highest ratio of calli induction and compact calli formation on MS medium supplemented with 1 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BA (96.67%) after 4 weeks of culture. From created calli, somatic embryogenesis could be induced on MS medium supplemented with 1 mgL^{-1} TDZ or 1 mgL^{-1} TDZ and 0.5 mgL^{-1} NAA (96 and 92%) after 4 weeks of culture, respectively. The optimum medium for shoot proliferation was MS medium supplemented with BA 5-7 mgL^{-1}; ½ solid MS medium supplemented with 2 gL^{-1} activated charcoal and 0.5-4 mgL^{-1} NAA was the most effective for root induction of kamchatic plantain.

Acclimatization of micropropagated plants could be planted in plastic pots containing a mixture of decayed straw : rice husk ashes, (1:1, v/v), sand : soil (1:1, v/v) or soil, showed a high survival rate (93.3-100%) and the most seedlings grew normally.

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