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Affect of brassinosteroids on *in vitro* proliferation and vegetative growth of potato

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Abstract
Potato is an important tuber crop grown extensively for consumption as staple food or for industrial uses. Seed generally accounts as the costliest input in potato cultivation that is often marred by poor quality or infections. *In vitro* multiplication of potato constitutes one of the most lucrative alternate options to circumvent the tuber borne problems in vegetative propagation. Hormones have profound effect on growth and differentiation of *in vitro* cultures. In this study we have showed the effect of brassinosteroids on *in vitro* culture and the subsequent growth of potato. We found that number of nodes, number of branches, shoot length, number of leaves, culture survival were highest under cultures supplied with Gibberellic acid (GA) + Napthaleneacetic acid (NAA) + Epibrassinolide (EBL) at 0.5 µM + 0.1 µM +0.1 µM concentration mixture. Shoot initiation was also earlier when cultures were supplied with GA+NAA+EBL (0.5 µM + 0.1 µM + 0.1 µM). We conclude that by the study that the effect of EBL in comparison to GA+NAA, length of shoots, numbers of nodes and leaves were significantly higher when GA+NAA were supplemented with EBL.

Keywords: Brassinosteroids, *in vitro* culture, hormonal cross talk, potato, vegetative growth

Introduction
Potato is a short-day plant (Went, 1957) [1], a cool season crop (Ewing, 1981) [2] and a C_3 plant with a low light saturation point (Demagante and Vander Zaag, 1988) [3]. It is known as a herbaceous, succulent, dicotyledonous plant with alternate stolons underground and alternate leaves on the stem above ground; stems are about 30 – 100 cm tall. A potato plant can have three kinds of stems including sprouts (leafy stems), stolons and tubers. Tubers are underground, fleshy stems with eyes and they are suitable for ware, food processing, seed, animal feed and non-industrial use (Beukema and Van der Zaag, 1990; Struik and Wiersema, 1999) [4, 5]. A potato crop, grown from seed tubers goes through five different stages: sprout development stage, vegetative growth stage, tuber initiation stage, tuber bulking stage and finally the maturation stage (Rowe *et al*., 1993) [6]. Potato (*Solanum tuberosum* L.) is world's single most important tuber crop which grows in about 150 countries and plays a vital role in global food system. The tubers are highly nutritious with high concentration of energy, high quality proteins, minerals and vitamins comparable to other quality products of plant origin like cereals. The potato is a wholesome food with all the extremely important and necessary dietary constituents, which are needed for health and growth (Pushkarnath, 1978; Li, 1985; Burton, 1989) [7, 8]. Compared to other roots and tubers and also many cereals, potato tubers have a high ratio protein to carbohydrates with a high nutritional value of the protein (Shekhawat *et al*., 1994, 1999) [9, 10]. Among the major inputs in potato cultivation, seed comes out to be the costliest input accounting for about 50% of total expenditure in potato cultivation and loss in potato yield due to poor quality of seed cannot be compensated even with all the essential inputs put together (Kushwah and Singh, 2008) [11]. Due to progressive accumulation of viral diseases in seed potato stock, virus infiltration can reach up to 100% in 3-4 successive crop seasons resulting in almost half or one third yield (Khurana *et al*., 2003) [12]. Undoubtedly, major constraint towards limiting yield and productivity of potato is unavailability of good quality potato seed. It is estimated that the application of healthy potato seed tubers will lead to at least 30% increase in potato yield (Zarghami, 2001). Brassinosteroids (BRs) are a new class of phytohormome and now considered as the sixth group of hormones in plant. As BRs can affect plant elongation, cell division and vascular development influencing morphogenesis, the use of this plant regulator in plant biotechnology is promising as supplementation to medium. It comprise a specific class of low-abundance plant steroids of ubiquitous occurrence in plants (Fujioka 1999; Belkhadir and Chory, 2006)

[14, 15]. BRs induce a broad spectrum of responses (Clouse and Sasse, 1998) [16, 20]. However, stimulation of growth via cell elongation and cell division is a major biological effect of BRs (Zurek *et al*., 1994; Hu *et al*., 2000) [17, 18].

Materials and Methods

The present investigation was carried out at Plant Tissue Culture Laboratory and Research Field (Garden Section) in the Department of Plant Physiology, College of Basic Science and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar, Udham Singh Nagar (Uttarakhand). Pantnagar is situated at an altitude of 243.84 m above mean sea level. It falls under the subtropical zone and is situated in the tarai region at the foothills of Shivalik range of the Himalayas. Investigations were classified into four experiments as mentioned hereunder:

Performance of second and third generation of potato tuber seed cv Kufri Himalini in field procured from tissue culture laboratory of erstwhile Hill Campus (Ranichauri) of GB Pant University of Ag. & Tech. where these tubers were produced under the project funded by HM-NEH-MM-1(2006-2012).

Effect of storage duration on weight of potato tubers.

Monitoring the *in vitro* growth of potato shoot cultures in MS media with hormones.

Proliferation of potato shoot cultures by using brassinosteroid (epibrassinolide) under aseptic conditions.

Performance of Generation

Source of Material

The planting material (stored tubers of potato cv. Kufri Himalini) were procured from tissue culture laboratory of erstwhile Hill Campus (Ranichauri) of GB Pant University of Ag. & Tech, where these tubers were produced under the project funded by HM-NEH-MM-1(2006-2012). The experimental material used in present investigation comprised of two samples of total 306 mature tubers of potato of second and third generation.

Experimental design and layout

Mature tubers were sown in the garden section of Plant Physiology Department, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar in January 2013. All the 306 tubers of 2 samples (2^{nd} and 3^{rd}) generations were sown in 3 long ridges per plot containing 17 tubers per ridges. Total 6 plots were made. Micro-tubers produced in the laboratory when transferred to field for the first time for mini-tuber production were termed as first generation and, thereafter, when multiplied in field for the second and third time were designated as 2^{nd} and 3^{rd} generation seed tubers.

Table 1: Generations of tubers used during experiment

Generations	Abbreviation
G(II)	F1(4-10g)
G(II)	F2(10-20g)
G(II)	F3(20-30g)
G(III)	F4(4-10g)
G(III)	F5(10-20g)

* F1, F2, F3, denoted as tubers of different weight of 2^{nd} generation and F4, and F5 denoted as tubers of different weight of 3^{rd} generation, respectively.

The field was prepared by ploughing up to a depth of 20 cm. Thereafter clod breaking, leveling and planking was done. The beds were prepared by adding 1 inch thickness of well decomposed FYM and sand in the ratio of 1:1 on top of the bed.

Tubers sowing and harvesting

Mature tubers were sown on 4^{th} January 2013, maintaining 50 cm row to row distance and 10 cm tuber to tuber distance. All the recommended agronomic practices were followed during experimentation. Tubers were harvested in the month of April, 2013 during (April 22 to 25, 2013).

Stock solutions of hormones

Stock solutions of all hormones used in the present study had concentration of 40mg/100ml. Separate stock solution were prepared for Calcium Panthonate, GA, NAA by dissolving them in minimum amount of 0.1NaOH and then by making up the volume to100 ml with distilled water. Epibrassinolide (0.4mg) was directly dissolved in 50% ethanol and the volume was made up to 100ml with distilled water.

All stock solutions were stored at 4^0C in small containers.

Culture Medium

Murashige and Skoog (1962) [19] medium was used as basal medium and supplemented with different plant growth regulators and vitamins as per the requirement of individual experiments. The culture medium thus prepared was autoclaved at 121°C and 15 psi for 20 min duration.

MS medium supplemented with hormones was used for establishment of cultures. Shoot proliferation from single and double node segment was studied by using different concentration of epibrassinolide, GA and NAA. Plantlets were inoculated in Murashige and Skoog medium supplemented with hormones for further proliferation of the shoots.

Table 2: Composition of Murashige and Skoog Medium used during proliferation

Components	Murashige and Skoog Medium (mg/l)
Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$CaNO_3$	-
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.8
Fe Citrate	-
Micronutrients	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Thiamine. HCl	0.1
Nicotinamide	0.5
Pyridoxine. HCl	0.5
Others	
Glycine	2.0
Sucrose	30,000

For establishment of explant

The culture medium was prepared by mixing individual

components from stock solutions and supplemented with calcium pantothenate, and different concentrations of GA, NAA to study proliferation and regeneration. Different treatments of GA+NAA are shown in Table 3.

Table 3: Different treatments of GA and NAA used for shoot establishment

Treatments	GA (µM)	NAA (µM)
T1	0.5	0.05
T2	0.5	0.1
T3	0.3	0.05
T4	0.3	0.1

For Shoot Proliferation

For shoot proliferation, best performing treatment T2 (0.5µM GA and 0.01µM NAA) was chosen from the experiment. This treatment was supplemented with different concentrations of epibrassinolide and used for media preparation and subsequent culturing. Different treatments of GA, NAA and epibrassinolide are shown in Table 4

Table 4: Different combinations of GA, NAA and Epibrassinolide for shoot proliferation

Treatments	GA (µM)	NAA (µM)	Epibrassinolide (µM)
A (control)	0.5	0.1	-
B	0.5	0.1	0.01
C	0.5	0.1	0.1
D	0.5	0.1	0.5

Statistical Analysis

The statistical analysis of all the parameters was done by using analysis of variance (ANOVA) and the data of all parameters was analyzed by ANOVA (analysis of variance) in accordance with simple CRD (Completely Randomized Design). The standard error of means (SEm) and critical difference (CD) were tested at the level of significance of $P \leq 0.05$.

Results and Discussion

Effect of Epibrassinolide on *in vitro* proliferation

Number of Nodes

On the basis of observations recorded in table 5, number of nodes were recorded at time interval (7, 14 and 21 days of establishment) under different combinations of hormones *viz*., A (0.5µM GA+0.1µM NAA), B (0.5µM GA+0.1µM NAA+0.01µM EBL), C (0.5µM GA+0.1µM NAA+0.1µM EBL) and D (0.5µM GA+0.1µM NAA+0.5µM EBL). After seven days of implantation, the highest number of nodes was recorded under the C (0.5µM GA+0.1µM NAA+0.1µM EBL) combination. After fourteen days of implantation, significantly higher (5.8) number of nodes were observed in C combination which was *at par* with A. The lowest value (1.5) was recorded in D combination. Number of nodes after twenty one days was found highest in C combination (8.5) which was significantly higher than other combinations.

Table 5: Effect of different concentration of Epibrassinolide on number of nodes

GA+NAA+EBL(µM)	After 7 days	After 14 days	After 21 days
A (0.5 GA/0.1 NAA) Control	2.8±0.48	4.3±0.63	6.5±0.65
B (0.5+0.1+0.01)	2.0±0.82	3.0±1.08	5.0±1.00
C(0.5+0.1+0.1)	4.3±0.63	5.8±0.85	8.5±1.26
D(0.5+0.1+0.5)	1.0±0.41	1.5±0.65	4.0±0.41
		Treatment (A)	Days (B)
	SEM ±	0.45	0.39
	CD at 5 %	1.29	1.12

Number of Branches

A glance of data presented in table 6 indicated that number of branches recorded after 7 days of implantation, in which A (0.5µM+0.1µM) and C (0.5µM+0.1µM+0.1µM) combination are similar with rest of the treatments likewise, combination B (0.5µM+0.1µM+0.01µM) and D (0.5µM+0.1µM+0.5µM) are significantly lower among other treatments. After fourteen days of implantation A and C combination are significantly similar among other combination. Number of branches recorded in hormonal combination A (0.5µM+0.1µM) and C (0.5µM+0.1µM+0.1µM) were similar. than other combination after twenty one days and in combination B (0.5µM+0.1µM+0.01µM) and D (0.5µM+0.1µM+0.5µM) number of nodes were significantly lower.

Table 6: Effect of different concentration of Epibrassinolide on number of branches

GA+NAA+EBL(µM)	After 7 days	After 14 days	After 21 days
A (0.5 GA/0.1 NAA) Control	1.0±0.00	1.8±0.25	2.0±0.41
B (0.5+0.1+0.01)	0.8±0.48	1.0±0.00	1.5±0.29
C(0.5+0.1+0.1)	1.0±0.41	1.8±0.48	2.5±0.29
D(0.5+0.1+0.5)	0.5±0.29	1.0±0.00	1.3±0.25
		Treatment (A)	Days (B)
	SEM ±	0.18	0.15
	CD at 5 %	0.51	0.44

Shoot Length

Data regarding effect of Epibrassinolide on shoot length of plantlets in culture vessels was also recorded (Table 7). It was evidented from the result that hormonal combination C (0.5µM+0.1µM+0.1µM) and A (0.5µM+0.1µM) showed significant similar result in shoot length after 7 days of implantation same as with fourteen days of implantation. In combination B and D the shoot length were significantly lower than other combination. The shoot length of plantlets was recorded significantly higher (4.7) in hormonal combination C as compared to other combinations after twenty one days of implantation. Lowest shoot length was observed in D combination after 21 days of implantation.

Table 7: Effect of different concentration of Epibrassinolide on shoot length

GA+NAA+EBL(µM)	After 7 days	After 14 days	After 21 days
A (0.5 GA/0.1 NAA) Control	2.0±0.41	2.5±0.65	3.2±0.66
B (0.5+0.1+0.01)	1.5±0.29	2.0±0.41	3.1±0.69
C(0.5+0.1+0.1)	2.5±0.29	2.8±0.25	4.7±0.48
D(0.5+0.1+0.5)	1.4±0.24	1.5±0.29	2.0±0.29
	Treatment (A)	Days (B)	AxB
SEM ±	0.25	0.22	0.44
CD at 5 %	0.73	0.63	1.26

Number of leaves

The number of leaves recorded at weekly interval showed that highest leaves were observed in hormonal combination C (0.5µM+0.1µM+0.1µM) combinations which were significantly higher among all other combination. After seven, fourteen days of implantation, lowest number of leaves were recorded in B and D combination, After twenty one days of implantation C combination and A combination showed significantly similar results as compared to rest of the treatments.Though the leaf size was not optimum due to shorter duration of growth but the leaves were normal morphologically (Table 8).

Table 8: Effect of different concentration of BR on number of leaves

GA/NAA/BR (µM)	After 7 days	After 14 days	After 21 days
A (0.5µM/0.1µM) Control	4.5±0.65	7.0±0.91	9.0±0.91
B (0.5µM/0.1µM/0.01µM)	3.0±0.41	5.0±0.41	6.0±0.82
C (0.5µM/0.1µM/0.1µM)	6.0±0.82	9.0±1.08	11±1.29
D (0.5µM/0.1µM/0.5µM)	3.0±0.41	3.8±0.75	5.5±0.65
		Treatment (A)	Days (B)
	SEM ±	0.46	0.40
	CD at 5 %	1.32	1.15

Shoot Initiation

Shoot initiation was also investigated under different combinations of hormones with in 5 and 10 days of time interval. It was observed that shoot initiation was started early at 5 days of time interval in C combination followed by other combinations.

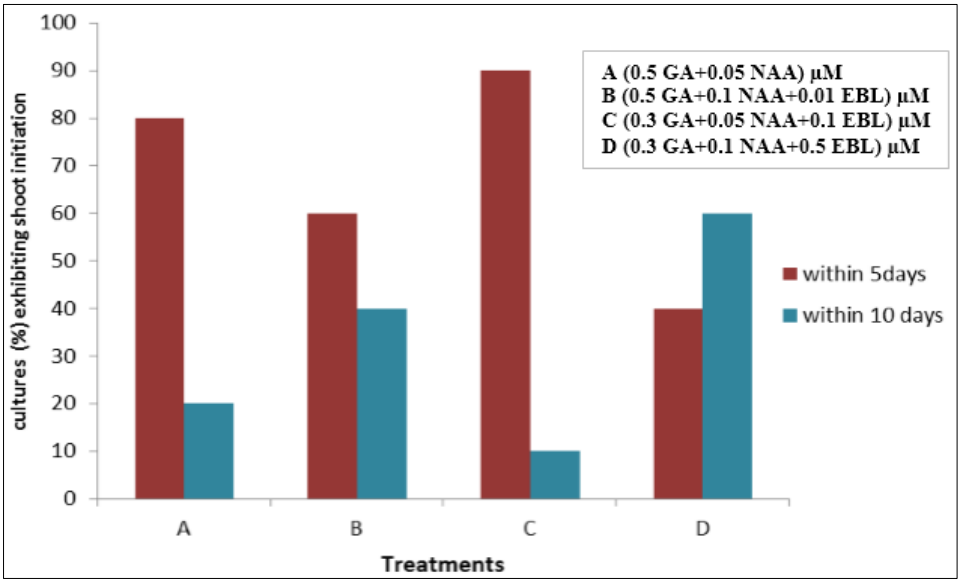

Fig 1: Effect of different concentrations of Epibrassinolide on shoot initiation

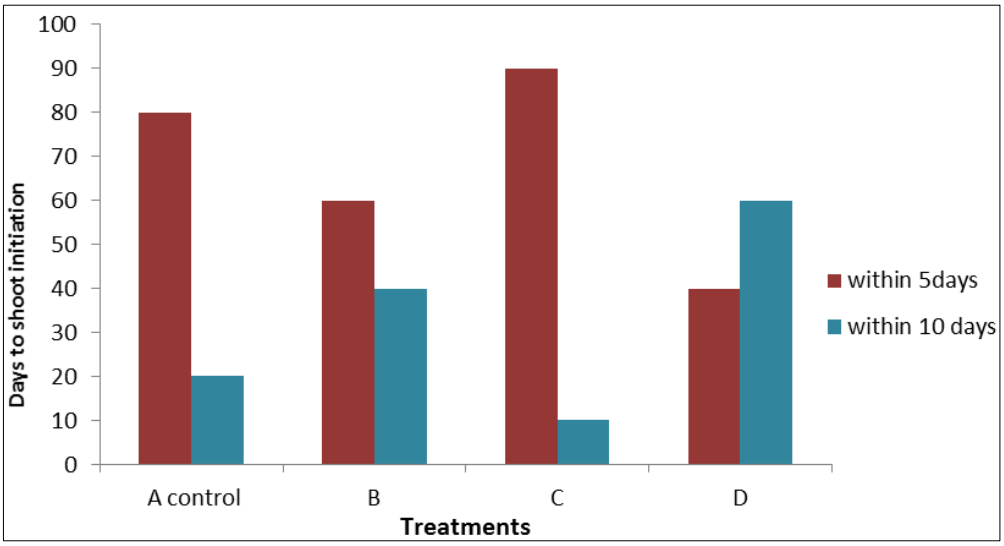

Fig 2: Effect of different concentrations of Epibrassinolide on days of shoot initiation

Culture Survival

Under different combinations of hormones, the maximum per cent of culture survival was observed in hormonal combination C at different time interval. During 1st week, 90% of culture survival was recorded in all combinations but as the weeks passed survivability of culture decreased in every combination but culture survival was maximum in C.

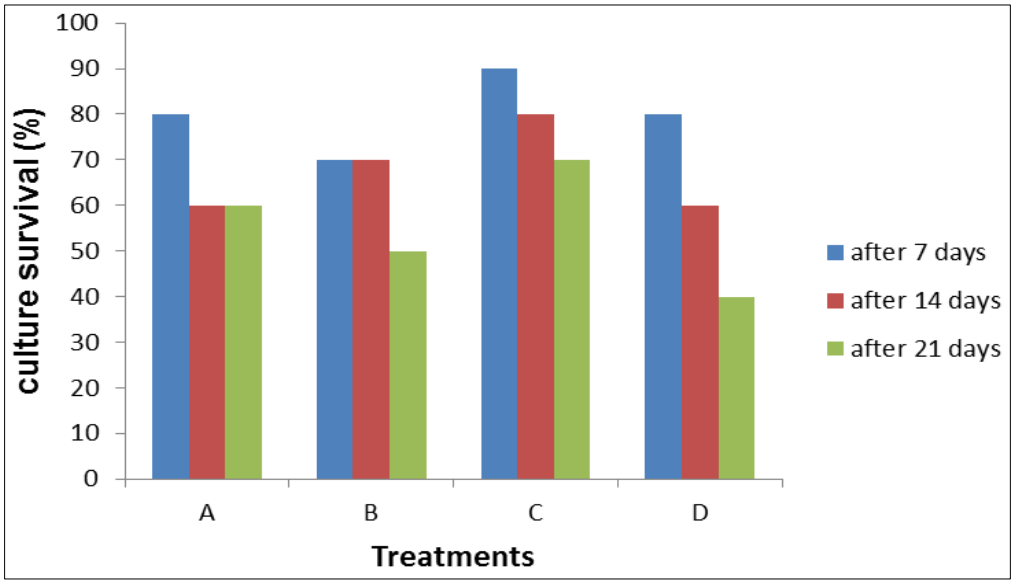

Fig. 3: Effect of different concentrations of BR on culture survival

Shoot proliferation of potato was the best in 0.1µM concentration of Epibrassinolide +0.5 µM GA. low amounts of commercial brassinosteroid enhanced shoot fresh wt. of cultured plantlets of *Solanum* sp. This result supports previous research which showed that brassinosteroids promote cell division and cell elongation, especially in young vegetative tissues Clouse and Sasse, (1998) [16, 20].

The response of brassinosteroid concentration in potato cultures was in general agreement with previously reported findings by a number of investigators using different concentration of brassinosteroids (Homobrassinolide). Some researchers reported that minor changes in the brassinosteroid concentration can strongly modify morphological responses, such as cell elongation. The physiological basis for this increase is still unknown, although many processes can be affected, as cited earlier. Similarly, the brassinosteroid (24-epibrassinolide) was used successfully to initiate protocorn like bodies and successful regeneration of *Cymbidium elegans* (Ravindra *et al*., 2007) [21]. Recently Gomes (2010) has also reported synergistic effects between auxin and BRs when BRs were used as a constituent in culture medium in plant tissue culture experiments.

These findings also support our observations regarding effect of epibrassinolide in enhancement of nodes, leaves, shoot length during the *in vitro* proliferation of potato cv. Kufri Himalini. The features of enhancement in number of nodes, leaves and shoot length with the inclusion of BRs along with GA+NAA in growth medium can further be exploited during microtuber production.

Khripach *et al.* (1999, 2000) [22, 23] further reported the growth and development of potato cuttings in culture medium containing brassinolide, 24-epibrassinolide, or 28-homobrassinolide.

Others studies have pointed out that BRs increase the numbers of vegetative and floral buds in other cultivars with diverse morphologies (Khripach, *et al.*, 1999) [22].

Concluding the results, amongst all the parameters taken to study the effect of EBL in comparison to GA+NAA, it was

revealed that length of shoots, numbers of nodes and leaves were significantly higher when GA+NAA were supplemented with EBL.

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