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Affect of naphthaleneacetic acid and gibberellic acid on in vitro proliferation and vegetative growth of potato in different combinations

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Abstract
Infected planting materials and slow rate of propagation are key factors limiting the yield and quality of potato. This study evaluated the effects of brassinosteroids, a a class of steroidal phytohormones on *in vitro* propagation of potato in 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). The tuber yield was found to be independent of number of eyes and seed size. Tuber weight was found to be inversely proportional to percent loss of weight with time. Number of nodes proliferated, number of branches and shoot length arising from tissue culture was found highest in treatment receiving higher GA:NAA (Gibberellic acid: Naphthaleneacetic acid in 0.5:0.1 µM concentration). Higher percentage of culture survival was observed with the aforementioned ratio of GA:NAA and similar results were obtained for shoot initiation from culture.

Keywords: potato, *in viro* multiplication, gibberellic acid, napthaneneacetic acid, vegetative growth

Introduction
The potato (*Solanum tuberosum)* originates from South America, most likely from the central Andes in Peru and introduced in India by the Portuguese in the seventeenth century. The potato was domesticated and has been grown by indigenous farming communities for over 4,000 years. The crop subsequently was distributed throughout the world, including Asia. (Shekhawat and Ezekiel, 1999) [1]. 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. In India, potato is popularly known as 'The king of vegetables' and has emerged as fourth most important food crop after rice, wheat and maize. In world scenario, India stands the second largest producer of potato (Scott and Suarez, 2011) [2]. Though, during the recent past, the productivity of potato in India has registered noticeable increase, but can this level be sustained or enhanced in future as per the requirements of the population, is a matter of concern today (Saxena and Mathur, 2013) [3]. 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) [4]. 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) [5]. In spite of having tremendous potential for augmenting potato production owing to varied agro-climatic conditions, potato growers in India suffer heavy losses due to (i) lack of scientific knowledge on seed potato technology, certification and supply systems (ii) lack of storage facilities, and (iii) lack of marketing and processing chain systems.
Micropropagation technique can be used to produce healthy seeds at right time because it is a unique method for the production of evenly propogated and disease free plants. Higher rate of multiplication, disease-free material (including plantlets, transplants, microtubers and minitubers), more convenient storage and transport, and a small demand for space during multiplication are known as advantages of rapid multiplication. Moreover, multiplication can be done whole year round (Wang and Hu, 1982; Marinus, 1983; Struik and Wiersema, 1999) [6, 12]. In addition, *in vitro* methods can be used for conservation, storage and easy distribution of potato germplasm in the form of breeding lines, new varieties and microtubers. *In vitro* tuber can be produced throughout the year. The potential value of tissue culture in potato production has been widely recognized (Wang and Hu, 1982; Badoni and Chauhan, 2009) [6, 7]. This technique is now widely used in many countries

(Murashige, 1974; Hussey and Stacey, 1981) [19, 20]. Rapid multiplication is very flexible and gives a high rate of multiplication. It also provides seed potato tubers free from seed borne diseases (Roca *et al*., 1978; Wang and Hu, 1982; Jones, 1988; Beukema and Van der Zaag, 1990) [21, 22, 23, 24]. Rapid multiplication can solve some of the problems associated with the conventional multiplication system (Lommen, 1995) [25]. Tissue culture revolutionized seed potato production in some countries such as Vietnam and doubled potato yield and potato area (Uyen and Vander Zaag, 1983, 1985) [26, 27]. A large number of virus-free potato microplants can be obtained by the micropropagation technique (Khurana *et al.,* 2003) [28].

Material multiplied in vitro has eventually to be transplanted into poly/glasshouses in order to produce clean mini tubers which possess distinct advantages. Though a lot of work has been done in India for standardizing in vitro propagation techniques using different hormonal combinations (GA/NAA) and a protocol for *in vitro* regeneration of Kufri Himalini has also been standardized by (Kumar and Kumar, 2012) [13].

Materials and Methods

Site description

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.

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 (2nd and 3rd) 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 2nd and 3rd generation seed tubers.

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.

Culture Medium

Murashige and Skoog (1962) 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.

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 3.3.

Table 1: 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.

Table 2: 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

* F1, F2, F3, denoted as tubers of different weight of 2nd generation and F4, and F5 denoted as tubers of different weight of 3rd 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. Mature tubers were sown on 4th 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). The observations were recorded by taking average plant height by using centimeter scale, number of shoots per tuber, number of tubers harvested per plant. After harvesting total yield was calculated from each plot where tuber seeds of 2nd and 3rd generations were sown.

Establishment of shoots

Surface sterilized nodal explants were carefully implanted on MS medium supplemented with calcium pantothenate and different combination of plant growth regulators. Culture bottles were then transferred to the culture room at 20-25°C and with 16/8 hour light and dark periods at 3000 lux light intensity. The observations were taken after 1, 2, 3 and 4 weeks for % of cultures having different number of branches, nodes, leaves, shoot length.

During proliferation

The best combination of hormones with MS medium was selected on the basis of the growth. Subculturing was done on medium supplemented with different concentrations of epibrassinolide, by cutting in to small pieces in a way that each subsection had 2 nodes and were grown in 10 replicates.

During subculturing
During subculturing, proliferated shoots were cut into double node segments in sterile petri dish in laminar air flow cabinet and propagated on culture medium supplemented with epibrassinolide of different concentrations, so as to represent three treatments for shoot proliferation. Double node segments were aseptically transferred into the culture tubes containing 10-12 ml culture medium. The tubes were incubated for further proliferation under 3000 lux and 16 hours photoperiod for 15-20 days.

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
The present investigation was divided in four experiments. First experiment was conducted in field to compare the performance of tubers of 2nd and 3rd generations of cultivar Kufri Himalini and their effect on plant growth and yield potential. The second experiment was conducted to study the weight loss of stored tubers subsequently for 8 months. Third experiment was conducted to monitor the *in vitro* growth of potato shoot cultures in MS medium with GA and NAA. Fourth experiment was done to study the effect of brassinosteroid (Epibrassinolide) on *in vitro* proliferation.

Field performance of tubers of 2nd and 3rd generation
Observations for average weight, number of eyes in different grades of seed tubers. Before sowing, mature tubers were divided into 5 grades (F1, F2, F3, F4, F5) on the basis of individual tuber weight. In which F1, F2, F3 grade tubers were of 2nd generation, whereas, F4 and F5 tubers were of 3rd generation. Observations recorded for tuber weight and number of eyes are presented in Table 3.

Weight of tubers (g)
F1, F2, F3 tubers of generation 2nd were categorized on the basis of average weight of tubers in the range of 4-10, 10-20, 20-30g and it was found to be 7.4, 14.6, 24.1g, respectively. F4 and F5 tubers of 3rd generation were categorized on the basis of weight ranges from 4-10 and 10-20g and it was found to be 4.2 and 14.3g, respectively.
The data in table 3 revealed that the highest weight (24.1g) among 2nd generation was observed in F3, which was significantly higher over all other grades. The minimum tuber weight (4.2g) was recorded in F4 of 3rd generation.

Number of eyes
The analyzed data presented in table 3, revealed that maximum numbers of eyes (2.00) were recorded in F3 which was higher over other grades but F2, F4, F5 had similar values while the minimum was recorded in F1.

Table 3: Effect of seed size and number of eyes on tuber yield

Grades	weight (g)	No. of eyes	Tuber Yield (number)
F1 G (II)	7.4±0.58	1.40±0.22	3.7±0.33
F2 G (II)	14.6±0.82	1.60±0.16	4.6±0.45
F3 G (II)	24.1±0.78	2.00±0.14	5.3±0.65
F4 G (III)	4.2±0.73	1.60±0.16	5.6±0.76
F5 G (III)	14.3±1.15	1.60±0.22	7.8±0.81
SEM±	0.68	1.53	0.52
CD at 5%	1.90	4.3	1.47

Comparison of yield
Comparison of F1 (GII) with F4 (GIII) and F2 G (II) with F5 (GIII) for response of average weight of seed towards tuber yield indicated that GIII was better than GII.

Effect of seed size on tuber yield
The values of seed weight and tuber yield are depicted in bar diagram (Fig.1). Though the number of tubers (7.8) in F5 was highest, F4 (smaller seed of GIII) was considered as best performer on the basis of fold increase in the number of tubers produced in comparison to seed size.

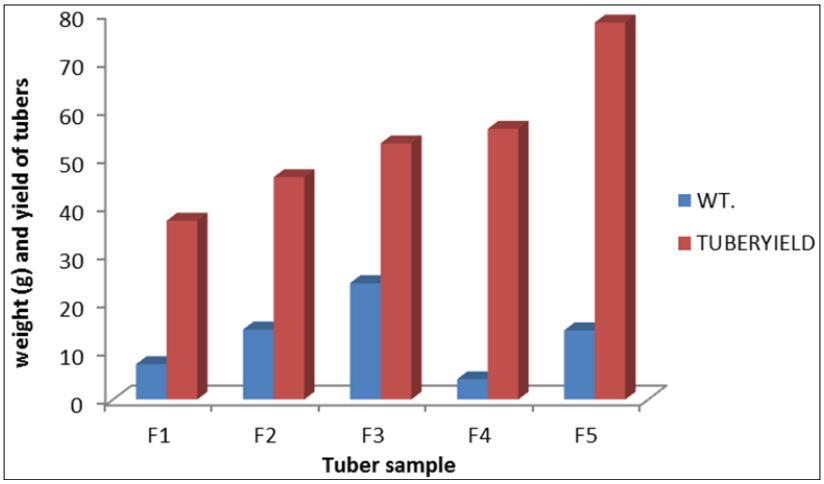

Fig 1: Relation between weight of seed and number of tubers

The data clearly indicated that smaller size tubers were also suitable for tuber production because the potential was found to be higher in 3rd generation than 2nd generation. Generation wise potential increases up to 4, 5 generation and then gradually decreases. This is also supported by Kleinkopf, *et al.,* (1990) [10] that early generation seed potentially carries lower levels of certain diseases than later generation seed.

Effect of storage duration on weight of potato tubers

Thereafter, second experiment was conducted to study the storage behavior of 3 categories of tubers PT1 (≤15g), PT2 (15-25g), PT3 (≤25g) and the results are shown in table 4.

Table 4: Effect of storage duration on weight of tubers

Month	PT1 (≤15 g)	PT2(15-25 g)	PT3(25g≤)
May	13.1±0.51	21.2±1.58	29.4±1.85
June	13.2±0.62	21.3±1.62	29.2±1.83
July	13±0.67	21.1±1.56	29.3±1.79
August	12.8±0.70	20.5±1.54	28.1±2.08
September	12.5±0.74	20.4±1.48	26.7±2.56
October	12.2±0.80	19.6±1.38	26.7±1.97
November	11.2±0.58	18.7±1.30	25.6±1.91
December	10.7±0.49	17.9±1.45	24.6±1.91
SEM	0.63	0.39	1.10
CD %5	1.79	1.09	3.10
% of weight loss	22	18	17

It could be inferred from the observations recorded for weight loss during storage (table 2) that smaller the tuber size, more was the percent loss in weight of tubers. The decrease in tuber weight with the increase of time duration in storage is influenced by several factors like environmental conditions, process of respiration, storage condition, chemical reactions (converting sugar and starches to carbon dioxide (CO_2) and water and losing moisture) and also because of vapor pressure differences between the tubers and the surrounding air. Similar observations were reported by Burton, (1989) [11]; Struik and Wiersema, (1999) [12].

Hence, smaller sized tubers should not be stored for longer period and should be utilized for ensuing crop after suitable dormancy breaking treatments. Thus it will be possible to use the produce of winter season potato crop grown in plains (Sep-Oct-Nov to Jan-Feb-Mar) for the summer season crop grown in hills (March-Apr to July-Aug).

In vitro establishment of plantlets of potato cv. Kufri Himalini without using Brassinosteroid (Epibrassinolide)

Modification of *in vitro* techniques

The nodal segments as explants were initially taken for implantation in medium as has been recommended for in vitro culture of potato (Kumar and Kumar, 2011).

Shoot proliferation during establishment

Growth of established cultures was recorded in terms of number of nodes, number of branches and shoot length and 5 or 10 replications in each treatment were planted.

Number of nodes

The number of nodes recorded at time interval 5, 10 and 20 days of implantation under different combinations of hormones viz. T1 (0.5µM GA+0.05µM NAA), T2 (0.5µM GA+0.1µM NAA), T3 (0.3µM GA+0.05µM NAA) and T4 (0.3µM GA+0.1µM NAA). Among these treatments, T2 (0.5µM GA+0.1µM NAA) was found to be more effective than other treatments. The number of nodes observed significantly higher in T2 as compared to other treatments at different time intervals. Enhanced number of nodes were recorded at T2 in different time intervals viz. five days of implantation (2.6), ten days of implantation (2.8) and fifteen days of implantation (3.0) Table 3. Similar findings were reported by Espinoza *et al.,* (1992) [14] in which it was found that when nodal sections were inoculated on to a MS culture medium supplemented with 0.25 mg/liter GA and 2.0 mg/liter calcium pantothenic acid, the number of nodes increased six fold within 3-4 weeks.

Table 5: Effect of hormonal combination on number of nodes

GA+NAA(µM)	After 5 days	After 10 days	After 15 days
T1 (0.5+0.05)	0.8±0.20	1±0.00	1±0.45
T2 (0.5+0.1)	2.6±1.03	2.8±1.11	3±1.00
T3 (0.3+0.05)	0.6±0.24	0.8±0.37	0.8±0.37
T4 (0.3+0.1)	0.8±0.37	0.8±0.37	1.4±0.40
		Treatment (A)	Days (B)
	SEM ±	0.34	0.30
	CD at 5 %	0.98	0.85

Number of branches

Week wise growth of nodal segments and their branching pattern is depicted in Table 6. The number of branches was also observed under different combinations of hormones at different time intervals of 5, 10 & 20 days of implantation. Among all the treatments, T2 (0.5µM GA+0.1µM NAA) was found to be more effective than other treatments. The number of branches was recorded significantly higher in T2 combination of hormones. The number of branches was found to be increased in T2 hormonal combination as compared to other combinations of hormones at different time intervals viz. 7 days of implantation (1.4), 14 days of implantation (2.0) and 21 days of implantation (1.6).

Table 6: Effect of hormonal combination on number of branches

GA+NAA(µM)	After 7 days	After 14 days	After 21days
T1 (0.5+0.05)	0.8 ±0.37	0.8 ±0.37	1.0± 0.45
T2 (0.5+0.1)	1.4±0.24	2.0±0.55	1.6±0.51
T3 (0.3+0.05)	0.6±0.24	1.0±0.45	0.2±0.20
T4 (0.3+0.1)	0.8±0.37	0.8±0.37	1.0±0.45
		Treatment (A)	Days (B)
	SEM ±	0.20	0.17
	CD at 5 %	0.58	0.50

Shoot length

Shoot length of plantlets in culture vessels was estimated after subsequent weeks which ranged from 2-8 cm. It was noted from the results that T2 combination of hormones showed significant increase in shoot length. The shoot length of plantlets was recorded higher in T2 hormonal combination as compared to other combinations of hormone at different time interval viz. five days of implantation (1.4), ten days of implantation (2.6) and fifteen days of implantation (3.2). The other combinations of hormones were less effective as shown in table 4.6. Similar study was done by Badoni and Chauhan, (2009) who reported that shoot height in M.S. medium with GA_3 and NAA combination showed better result in comparison to M.S medium with Kinetin and NAA. Yousef *et al*., (1997) had used hormone NAA and GA_3 in medium and reported longest main shoot and highest node number.

Table 7: Effect of hormonal combination on Shoot length

GA+NAA(µM)	After 5 days	After 10 days	After 20 days
T1 (0.5+0.05)	0.7±0.31	1.4±0.40	2.1±0.56
T2 (0.5+0.1)	1.4±0.37	2.6±0.75	3.2±0.86
T3 (0.3+0.05)	0.8±0.37	1.7±0.30	1.6±0.75
T4 (0.3+0.1)	0.5±0.32	1.2±0.58	1.5±0.63
		Treatment (A)	Days (B)
	SEM ±	0.31	0.27
	CD at 5 %	0.90	0.78

Culture Survival

Under different combinations of hormones, the maximum per cent of culture survival was observed in T2 combination at different time interval. During 1st week 100% of culture survival was recorded in all combinations but as the weeks passed survivability of cultures decreased in every combination but it was maximum in T2 (fig. 2).

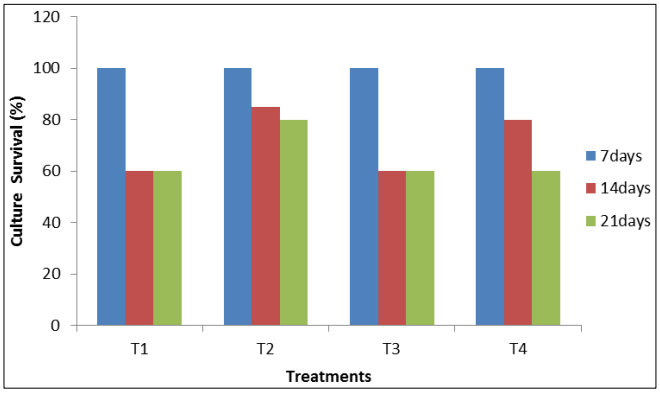

Fig 2: Effect of different combination of hormones in culture survival

Shoot Initiation

Shoot initiation was also investigated under different combinations of hormones within 5 and 10 days of time interval (Fig 3). It was observed that shoot initiation started early at 5 days of time interval in T_2 combination followed by other combinations. Among all the treatments, T_2 combination showed significant increase in shoot initiation at 5 days of time interval.

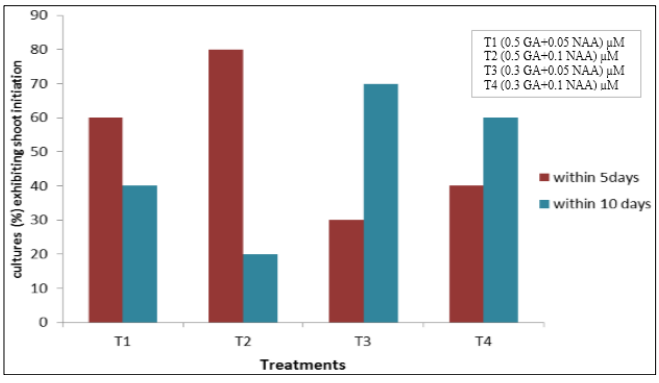

Fig 3: Effect of different combination of hormones in shoot initiation

Results obtained in present investigation were in accordance with the work of Badoni and Chauhan, (2009). They reported that use of GA + NAA (0.25 mg/l GA and 0.01 mg/l NAA) combination was best for shoot regeneration and multiplication of potato cv. Kufri Himalini in comparison to the combination Kinetin + NAA with M.S medium. Lowest shoot height and number of nodes was observed in other combination of GA and NAA, (0.25 mg/l GA_3 and 0.03 mg/l NAA) and (0.25 mg/l GA and 0.04 mg/l NAA) this may be due to higher concentration of NAA. Similar observations were also recorded by Pennazio and Vecchiati, (1976) that higher concentration of NAA inhibited root and shoot growth. Results showed that lower concentration of auxin (0.01 mg/l NAA) with gibberelic acid (0.25 mg/l GA) seems to be the best for development of complete plantlets. Similar findings were also reported by Moeinil *et al.* (2011) that maximum growth of potato plantlets was obtained on MS solid medium with 0.25 mg L^{-1} GA_3 and 2 mg L^{-1} calcium pantothenate.

MS liquid medium supplemented with 0.25 mg L^{-1} gibberellic acid and 5.0 mg L^{-1} pantothenic acid is regarded as the most suitable regime for potato micropropagation. Undoubtedly, *in vitro* multiplication is a good technique to use with nodal segments to produce plants/ micro/ mini tubers thereby saving the conventional tubers as a seed.

The T2 combination of GA + NAA showed best result with reference to all the parameters studied during this course of investigation.

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