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COMBINED EFFECT OF CYTOKININ AND SUCROSE ON *IN VITRO* TUBERIZATION PARAMETERS OF TWO CULTIVARS i.e., DIAMANT AND RED NORLAND OF POTATO (*SOLANUM TUBEROSUM*)

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Abstract

The present study is focused on the optimization of culture media and explant density of apical and nodal segments of two potato cultivars for *In vitro* microtuber production. Fifty media manipulations of Murashige & Skoog (1962) were tested by varying concentrations of sucrose (4, 6, 8, 10 and 12%) either alone on in combination (5-8% sucrose) with kinetin and BA. Different parameters with regard to microtuber induction mean number of microtubers, mean fresh weight of microtubers both from single and multinode explant of Diamant and Red Norland were studied. Medium MB8 (5mg BA and 8% sucrose) in term of induction and mean number of microtubers per single node explants and MB11 (6mg BA and 7% sucrose) in terms of mean fresh weight per single node provided best results for cv. Diamant. In case of Red Norland, medium MK10 (2mg Kinetin and 6% sucrose) in term of induction, mean number and mean fresh weight of microtubers per multinode explants gave best results. In comparison, cv. Diamant was better for microtuber induction and development than cv. Red Norland.

Introduction

Potato is the fourth most food crop after wheat, rice and maize, therefore, the most important dicotyledonous and tuber crop (Jones, 1994). Being developing country and having limited resources the disease free potato production in Pakistan is only 4%. Seed tubers are the most common source of plant material in potato reproduction. Potatoes, with the conventional method of vegetative propagation are often prone to attack by pathogens such as fungi, bacteria and viruses, thereby resulting in poor quality and yields. Recently, plant tissue culture technology has become very popular and has a visible impact on the production of virus free seed potatoes. Evidence for strong and consistent analogies between microtubers and field grown tubers for their induction, growth and development, several components such as the rapid and near synchronous induction and growth, which can be modified by a range of exogenous compounds or conditions, make the microtuber a valuable model system (Coleman *et al*., 2001). Microtuber production is one of the strategies under this perspective. Because of their small size and weight microtubers have tremendous advantages in terms of disease free, storage, transportation and mechanization (Kefi *et al*., 2000b; Kanwal *et al*., 2006). A number of research groups all over the world are trying to bring about this revolution (Gopal *et al.,* 2004; Zhijun, *et al.,* 2005; Zhang, 2006). Now a days, exogenous supply of cytokinin and cytokinin like compounds in microtuber growth media has been getting much attention for future perspective (Shibli *et al.,* 2001). However, cytokinin stimulated transition of axillary buds into stolons, which could be useful in tuberization *In Vitro* but not maintenance of shoot cultures (Vinterhalter *et al*., 1997).

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The objective of present study was to produce virus free *In vitro* microtubers in terms of induction time, mean number, mean fresh weight of microtubers by varying explant density i.e, single node and multinode explants and evaluation of genotypic responses of two potato cultivars.

Materials and Method

The healthy virus free potato tubers were obtained from Punjab University Seed Centre. These tubers were washed several times with detergent followed by several times rinsed with distilled water, dried and placed in dark room for eight week till sprouting started. One week old sprouts were dipped in 15% NaOCl solution for 15-20 minutes, given three washings with autoclaved distilled water and inoculated on prepared MS medium. After 4 weeks of inoculation the buds were sprouted into full plantlets that contained 7-8 nodes. These were excised into singlenode (one node) and multinode (three nodes) explants and used for microtuberizaton experiments. The MS media used was supplemented with sucrose (4, 6, 8, 10 & 12%) either alone or in combination with BA (6-benzylaminopurine) and Kinetin (6-furfurylaminopurine) at varying concentrations. The pH of the medium was adjusted at 5.74. In each test tube 10 ml media was dispensed and capped before autoclaving. The media was autoclaved at 121°C for 15 minutes under the pressure of 15 Ib/In2. After inoculation the vials were transferred to growth room where temperature was kept at 27°C ± 1°C and 16 hour day light. When microtubers become matured they were harvested into sterilized Petri plates aseptically. These Petri plates were sealedwith Nesco film and stored at 4°C for 3 months.

Results

Effect of sucrose on microtuberization: The results of microtuber induction in cultivars Diamant and Red Norland on MS medium supplemented different concentrations of sucrose i.e. 4, 6, 8, 10 and 12% without any growth regulator showed that the medium M4 containing 8% sucrose proved to be optimal in terms of minimum time of induction (21 & 18 days), mean number (1.4 & 1.8) and fresh weight (0.04 & 0.10 g) of microtuber per single and multinode explant, respectively in cv. Diamant (Table 1). For cv. Red Norland in the medium M8 containing same concentration of sucrose (8%), the minimum time of induction (36 & 40 days), mean number (0.9 & 1.8) and fresh weight (0.03 & 0.04 g) of microtuber per single and multinode explant, respectively, was optimized.

Effect of cytokinin and sucrose on microtuberization: Effect of different concentrations of Kinetin ranging from 2.0 and 2.5 mg/l with high concentrations of sucrose (5-8 %) were also studied for microtuberization. It was observed that 2.5 mg/l of kinetin with high concentrations of sucrose gave better response. Best results of this combination were achieved when 2.5 mg/l kinetin was used with 8% sucrose in MS medium (MK8) for cv. Diamant. At this concentration microtuber induction started after 14 & 12 days of inoculation and maximum 2.1 & 2.9 microtubers per single and multinode explant, respectively. The maximum mean fresh weight of 0.23 & 0.14 g per single node and multinode explant, respectively, was observed in medium MK7. For cv. Red Norland the best results was found in the medium MK10 containing sucrose concentration of 6%, the minimum time of induction (30 & 28 days), mean number (1.1 & 2.5) and fresh weight (0.04 & 0.14 g) of microtuber per single and multinode explant, respectively (Figs. 3 & 4). In case of cv. Red Norland, Kinetin in combination with high sucrose gave microtubers of larger size per multinode explant as compared to other treatments (Fig. 6).

Table 1. Effect of sucrose concentrations on *In vitro* induction, mean number and mean fresh weight of microtubers from single and multinode explants in *Solanum tuberosum* L.

Cultivar	Media no.	Sucrose (%)	Single node explant			Multinode explant		
			Microtuber induction (days)	Mean no. of microtubers	Mean FW (g) of microtubers	Microtuber induction (days)	Mean no. of microtuber	Mean FW (g) of microtubers
	M1	4	40	0.7 ± 0.2	0.05 ± 0.03	37	0.9 ± 0.02	0.05 ± 0.02
	M2	6	36	0.8 ± 0.3	0.05 ± 0.02	29	1.3 ± 0.21	0.05 ± 0.02
Diamant	M3	8	21	1.4 ± 0.3	0.04 ± 0.03	18	1.8 ± 0.32	0.10 ± 0.03
	M4	10	28	1.3 ± 0.3	0.05 ± 0.02	19	1.7 ± 0.21	0.11 ± 0.04
	M5	12	29	1.2 ± 0.2	0.05 ± 0.02	22	1.7 ± 0.09	0.11 ± 0.18
	M6	4	73	0.6 ±0.2	0.02 ± 0.01	68	0.8 ± 0.09	0.03 ± 0.30
	M7	6	62	0.7 ± 0.1	0.02 ± 0.001	54	1.1 ± 0.21	0.03 ± 0.21
ed norland	M8	8	40	0.9 ± 0.2	0.03 ± 0.21	36	1.8 ± 0.47	0.03 ± 0.01
	M9	10	41	0.8 ± 0.4	0.03 ± 0.42	38	1.6 ± 0.56	0.04 ± 0.29
	M10	12	48	0.7 ± 0.5	0.04 ± 0.16	43	1.6 ± 0.44	0.03 ± 0.21

V= Fresh weight, ± Standard error of mean of ten replicates.

Table 2. Effect of Kinetin and sucrose concentrations on *In Vitro* induction, mean number and mean fresh weight of microtubers from single and multinode explants in *Solanum tuberosum* L.

Cultivar	Media No.	Kinetin (mg/l)	Sucrose (%)	Single node explant			Multinode explant		
				Microtuber induction (days)	Mean No. of microtubers	Mean FW (g) of microtubers	Microtuber induction (days)	Mean No. of microtuber	Mean FW (g) of microtubers
Diamant	MK1	2.0	5	19	1.7 ± 0.92	0.04 ± 0.02	16	2.3 ± 0.52	0.03 ± 0.04
	MK2	2.0	6	14	1.8 ± 0.86	0.11 ± 0.01	14	2.8 ± 0.83	0.05 ± 0.04
	MK3	2.0	7	15	1.9 ± 0.98	0.13 ± 0.03	13	2.4 ± 1.30	0.04 ± 0.02
	MK4	2.0	8	13	2.1 ± 0.35	0.14 ± 0.02	13	2.6 ± 0.82	0.05 ± 0.03
	MK5	2.5	5	18	2.1 ± 1.50	0.13 ± 0.04	17	2.8 ± 0.61	0.06 ± 0.04
	MK6	2.5	6	14	1.8 ± 1.10	0.17 ± 0.05	13	2.9 ± 0.54	0.12 ± 0.04
	MK7	2.5	7	16	1.9 ± 0.93	0.23 ± 0.04	15	2.5 ± 0.39	0.14 ± 0.03
	MK8	2.5	8	14	2.1 ± 0.52	0.19 ± 0.04	12	2.9 ± 0.41	0.13 ± 0.03
Red Norland	MK9	2.0	5	65	0.6 ± 0.21	0.03 ± 0.94	62	1.2 ± 0.96	0.05 ± 0.01
	MK10	2.0	6	30	1.1 ± 0.36	0.04 ± 0.32	28	2.5 ± 0.59	0.15 ± 0.02
	MK11	2.0	7	39	0:8 ± 0.29	0.04 ± 0.01	38	1.7 ± 0.63	0.14 ± 0.02
	MK12	2.0	8	42	0.8 ± 0.31	0.04 ± 0.06	39	1.8 ± 0.84	0.13 ± 0.01
	MK13	2.5	5	39	0.8 ± 0.33	0.05 ± 0.02	35	1.7 ± 0.29	0.14 ± 0.39
	MK14	2.5	6	43	0.9 ± 0.28	0.04 ± 0.04	38	1.9 ± 0.43	0.14 ± 0.24
	MK15	2.5	7	45	0.9 ± 0.41	0.04 ± 0.01	43	1.8 ± 0.51	0.13 ± 0.31
	MK16	2.5	8	38	1.0 ± 0.39	0.04 ± 0.02	34	2.3 ± 0.49	0.12 ± 0.20

FW = Fresh weight: ± Standard error of mean of ten replicates.

Table 3. Effect of BA and sucrose concentrations on *In vitro* induction, mean number and mean fresh weight of microtubers from single and multinode explants in *Solanum tuberosum* L.

Cultivar	Media No.	BA (mg/1)	Sucrose (%)	Single node explant			Multinode explant		
				Microtuber induction (days)	Mean No. of microtubers	Mean FW (g) of microtubers	Microtuber induction (days)	Mean No. of microtuber	Mean FW (g) of microtubers
Diamant	MB1	4.0	5	22	1.8 ± 0.80	0.06 ± 0.07	18	2.6 ± 0.50	0.043 ± 0.05
	MB2	4.0	6	19	1.7 ± 0.50	0.12 ± 0.40	16	2.9 ± 0.37	0.068 ± 0.04
	MB3	4.0	7	19	1.4 ± 0.30	0.06 ± 1.17	13	3.3 ± 0.39	0.048 ± 0.06
	MB4	4.0	8	18	2.5 ± 0.24	0.06 ± 0.93	12	3.2 ± 0.38	0.041 ± 0.01
	MB5	5.0	5	11	1.7 ± 1.10	0.10 ± 0.02	10	2.8 ± 0.72	0.082 ± 0.82
	MB6	5.0	6	10	1.8 ± 0.62	0.12 ± 0.04	9	3.3 ± 0.83	0.089 ± 0.29
	MB7	5.0	7	11	1.7 ± 0.30	0.10 ± 0.03	10	2.4 ± 0.43	0.062 ± 1.00
	MB8	5.0	8	10	2.8 ± 0.40	0.12±0.03	10	3.2 ± 0.41	0.113 ± 0.3
	MB9	6.0	5	21	1.6 ± 0.68	0.14 ± 0.60	15	2.9 ± 0.31	0.089 ± 0.29
	MB10	6.0	6	23	1.3 ± 0.83	0.19 ± 0.91	17	2.7 ± 1.40	0.101 ± 0.48
	MB11	6.0	7	16	1.4 ± 0.59	0.37 ± 0.08	14	2.5 ± 0.24	0.108 ± 0.09
	MB12	6.0	8	15	1.9 ± 0.54	0.28 ± 0.04	13	3.1 ± 1.10	0.112 ± 0.47
Red Norland	MB13	4.0	5	38	0.7 ± 1.10	0.03 ± 0.05	28	1.3 ± 0.80	0.046 ± 0.29
	MB14	4.0	6	35	0.8 ± 1.40	0.03 ± 0.04	35	1.4 ± 1.10	0.042 ±0.25
	MB15	4.0	7	28	1.1 ± 0.90	0.03 ± 0.02	26	1.7 ± 0.50	0.051 ± 0.04
	MB16	4.0	8	37	0.8 ± 0.90	0.03 ± 0.09	32	1.9 ± 0.40	0.047 ± 0.31
	MB17	5.0	5	30	0.9 ± 1.20	0.03 ± 0.04	28	1.6 ± 0.70	0.046 ± 0.38
	MB18	5.0	6	32	0.8 ± 1.30	0.03 ± 0.75	25	1.4 ± 0.40	0.052 ± 0.42
	MB19	5.0	7	33	0.9 ± 0.80	0.03 ± 0.16	30	1.7 ± 1.10	0.047 ± 0.51
	MB20	5.0	8	29	1.2 ± 0.40	0.03 ± 0.63	27	2.1 ± 0.30	0.054 ± 0.89
	MB21	6.0	5	29	0.8 ± 0.34	0.03 ± 0.89	28	1.9 ± 0.40	0.043 ± 0.62
	MB22	6.0	6	37	0.9 ± 1.20	0.03 ± 0.52	35	1.8 ± 0.90	0.039 ± 0.32
	MB23	6.0	7	29	1.1 ± 0.41	0.03 ± 0.29	28	2.1 ± 0.24	0.043 ± 0.07
	MB24	6.0	8	36	0.9 ± 0.80	0.03 ± 0.32	30	1.9 ± 0.42	0.048 ± 0.39

FW; Fresh weight; ± Standard error of mean of ten replicates.

As far as the combination of BA and high concentration of sucrose is concerned it was observed that low concentration i.e. 1.0 mg/l to 3.0 mg/l BA failed to show significant effect on microtuberization response. Best response for cv. Diamant was obtained in MB8 medium containing 5.0 mg/l BA with 8% sucrose (Figs. 1 & 2). At this concentration microtuber formation started after 10 days of inoculation for both single node and multinode explants. The mean number 2.8 & 3.2 microtuber, per single and multinode explant, respectively, was optimized. In case of cv. Diamant the maximum mean fresh weight 0.37 g of microtuber per single node explant (Fig. 5) was observed in medium MB11. For cv. Red Norland the optimum results in terms of minimum time of induction (29 & 27 days), mean number (1.2 & 2.1) and fresh weight (0.03 & 0.05 g) of microtuber per single and multinode explant, respectively was found in the medium MB20 containing 8% of sucrose .

Discussion

Microtuber induction: In comparison of both cvs. in term of microtuber induction (Tables 1, 2 &3), multinode explants were observed to be earlier *In vitro* tuberized than single node explant. It might be due to presence of some endogenous level of cytokinin in multinode explant than single node. Among media without any addition of cytokinin (Table 1), 8% sucrose level was found to be optimal for both cultivars. Khuri & Moorby (1995) proposed that the high sucrose level on one hand provides a good carbon source which was easily assimilated and converted to starch for the microtuber growth and on the other it secures an uninterrupted synthesis of starch due to high osmotic potential provided by the excess sucrose. At 8% sucrose provides a concentration favorable for the development of microtubers. Carlson (2004) and Sushruti *et al*., (2004) also reported best microtuber induction response ion MS medium supplemented with 8% sucrose. Miranda *et al.*, (2005) used MS medium without plant growth regulators to induce microtuberization by the addition of liquid MS medium supplemented with 8 g sucrose/litre. According to Nawsheen (2001) optimal production of microtubers was obtained in MS medium supplemented with 10% sucrose contents. Data presented in Table 1 shows that by further increasing the concentration of sucrose not only time taken for microtuberization was increased but mean number of microtubers per culture vial were also decreased both in single node as well as multinode explants.

The influence of combined action of cytokinin and higher sucrose (8%) level in cv. Diamant shortened the microtuber induction time from 21 & 18 days (8% sucrose alone) to 10 days when MS medium supplemented with BA (5mg/l) and sucrose (8%) per single and multinode explant, respectively. Aksenoa *et al.*, (2000) also reported that cytokinin and sucrose at high concentration stimulated tuber initiation. Like Diamant, the reduction in induction time was observed in cv. Red Norland but time delayed from 40 & 36 days (8% sucrose alone) to 29 & 27 days as compared to Diamant using same medium per single and multinode explant, respectively. Zakaria *et al*., (2008) obtained earliest microtuberization by 13 days at 10 mg/l BA in cv. Diamnt.

The onset of tuber initiation was significantly advanced by cytokinins as compared to control. Basal acid invertase activity in stolons cultured on control medium significantly increased form hook stage to swelling stage and then decreased slightly when tubers were initiated. At hook stage highest acid invertase activity occurred when segments were treated with cytokinin. The cytokinin stimulates enzyme activity early in the growth of stolons, which may have resulted in faster stolon growth and in earlier tuber initiation.

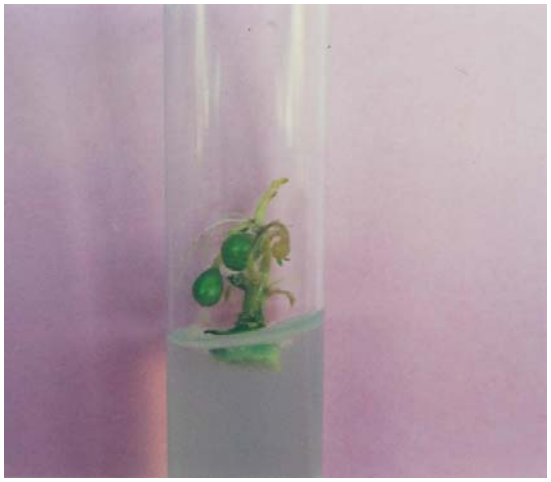

Fig 1. Three microtubers of cv. Diamant using single node explant on MS medium containing BA (5mg/l) and sucrose (8%).[1-X]

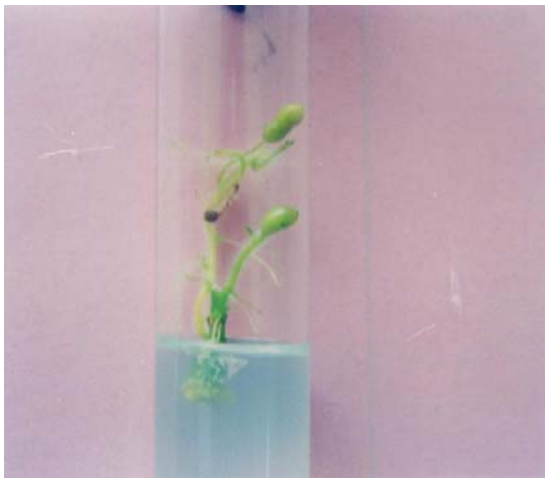

Fig. 2. Three microtubers of cv. Diamant using multimode explant on MS medium containing BA (5mg/l) and source (8%).[1-X]

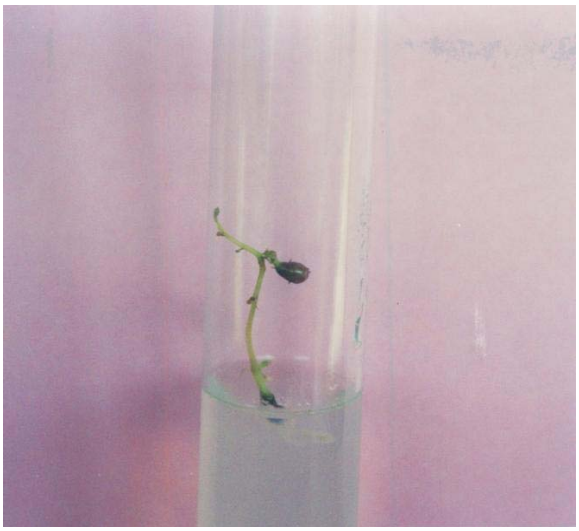

Fig. 3. One microtuber of cv. Red Norland using single node explant of MS medium containing Kinetin (2mg/l) and source (6%).[1-X]

Fig. 4. Two microtuber of cv. Red Norland using multimode explant of MS medium containing Kinetin (2mg/l) and source (6%).[1-X]

Fig. 5. One largest (0.370g) microtuber of cv. Diamant using single node explant of MS medium containing BA (6mg/l) and source (7%). [1-X]

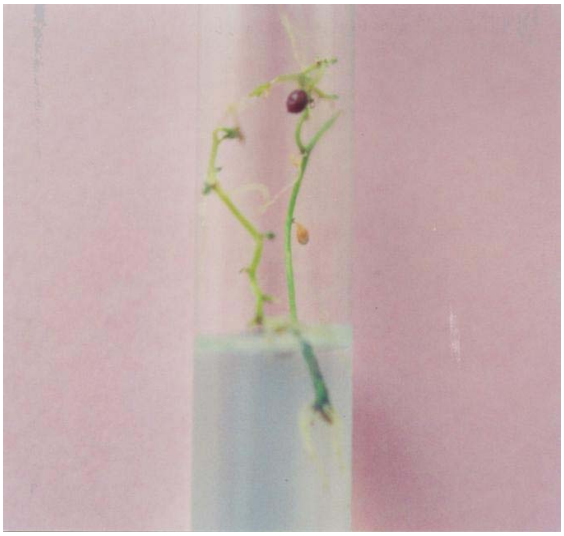

Fig. 6. One largest (0.15g) microtubers of cv. Red Norland using multinode explant on MS medium containing Kinetin (2mg/l) and source (6%).[1-X]

Mean number of microtubers: The higher mean number of microtubers was achieved by the combined effect of cytokinin and sucrose promotes *In vitro* microtuberization than sucrose alone (Tables 1, 2 & 3). The maximum 2.8 mean number of microtuber per single

node by using MS medium supplemented with BA (5mg/l) and sucrose (8%) in cv. Diamant. Jimenez (1999) obtained an average of 3.1 & 2.8 tubers per single node cutting of Desiree and Atlantic, two potato cultivars in a temporary immersion system by using 4l vessel. Rafique *et al*., (2004) achieved highest number of microtubers (0.69) per plant using 6% sucrose and 1 µM BA. Azzopardi (1997) used tuberization medium containing high level of BA (5.0 mg/l) and sucrose (8%) to get optimal production of microtubers. At same medium composition but with the addition of CCC (2-chloroethyltrimethylammonium chloride) in concentration of 500 mg/l, the maximum mean number of 44.5 microtubers per 100 ml flask was obtained by Haque (1996). Perl *et al.*, (1991) suggested that high (8%) sucrose levels could activate the class I B33 patatin promoter in tissues where it normally is not expressed. The BA at 14 mg/l in MS medium supplemented with 8% sucrose was found to be an optimum medium by Mogollon *et al.*, (2000). In contrast, maximum mean number (2.5) of microtuber was observed in cv. Red Norland by using MS medium supplemented with Kinetin (2 mg/l) and sucrose (6%). Aslam *et al*., 2007 reported 2.5 & 3.6 microtubers per single and multimode explant, respectively when MS medium was supplemented with Kinetin (2.5 mg/l) and sucrose (8%) in potato cv. Cardinal.

Mean fresh weight of microtubers: Single node explants were observed to give microtubers of larger size than multimode explant in cv. Diamant (Tables 1, 2 & 3). To obtain microtubers of larger sized the media supplemented with cytokinin and higher sucrose level were found to be optimal for both cultivars. The data representing in Table 3 the largest microtuber (0.37 g) per single node explant in cv. Diamant. According to Forti *et al.*, (1991) at low culture densities, the microtuber weight formed *In Vitro* increase and larger microtubers could be stored longer than smaller ones. In case of cv. Red Norland microtubers of 0.15 g per multinode explant were found as shown in Table 2. Pruski *et al*., (2001c) investigated that microtubers below 0.1 g were considered too small for green house production of minitubers (nuclear seed tubers). In comparison of two cultivars, microtubers were of larger sized in cv. Diamant than Red Norland. Size of microtubers was crucial for sprouting *In vivo*. It was suggested that only microtubers larger than 250 mg be used to produce minitubers *In Vivo* (Al-Safadi *et al*., 2000). According to Lecrerc *et al.*, (1995) smaller microtubers (≤250 mg) had longer dormants periods than did those greater than 250 mg.

Conclusion

From the results, it appears that cytokinin in combination with high sucrose promote *in vitro* microtuber induction and development. The cytokinins are stimulatory to starch metabolizing enzymes, thus creating a strong metabolic sink. As a result subsequent accumulation of starch occurred which is seen as the swelling of the microtuber. This combined ability can be termed as an excessive substrate (high sucrose level) and stimulus (BA or Kinetin) that triggers the enzymatic activity in the tuberization processes. The cultivar Red Norland is slow growing *in vitro* microtuberization experiments than cv. Diamant. The results suggested the need of developing genotype specific protocols to maximize *In Vitro* performance for microtuberization.

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