SHOOT-BUD FORMATION AND PLANTLET REGENERATION IN POTATO TUBER TISSUE CULTURED *IN VITRO*

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Introduction

Most of agronomical species which have been so far reported to initiate shoot-buds in culture *in vitro* followed by plantlet formation, examples of tobacco, carrot, endive, rape seed, *et c*, are the crops generally propagated by seed in which the propagation through tissue culture method has been of little interest. Much of practical and potential value in tissue culture techniques for cloning and mass propagation of outstanding genotypes of potato crops depends upon the possibility of developing effective and reproducible method of plantlet regeneration in potato tuber tissue, the quality of which is indispensable for restoration in regenerated potato crops.

The process of shoot-bud formation in potato tuber tissue cultured *in vitro* has only been reported to be feasible for two varieties, Russet burbank and Superior, while the isolation of protoplasts and a single cell *in vitro* and the subsequent recovery of plantlet have readily been accomplished in potato mesophyll cells[10]. However the data concerning the frequency of shoot-bud differentiation and plantlet regeneration in potato tuber tissue cultures are insufficient[3,5], and the potato, which is one of the four important food crops in world, has been difficult to achieve plantlet regeneration from explants other than shoot tips[11,13]. Development of techniques of plantlet regeneration through tissue culture of explant from storage organ is not only important for clonal multiplication after crop improvement but also essential for the studies on the process of cellular redifferentiation from differentiated cells.

In this paper, we report the results of studies on the plantlet regeneration in potato tuber tissue cultures *in vitro* research designed to establish culture conditions and controlling factors for shoot-bud formation in excised

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tuber tissue followed by plantlet regeneration.

Materials and Methods

Preparation of culture:

Discs (1×6 mm, in diameter) were excised from the central core of tubers of potato (*Solanum tuberosum* L., cv. Irish Cobbler potato) under aseptical conditions as described by OKAZAWA *et al.*[10], and cultured on the nutrient agar medium in 125-ml flasks in a growth chamber at 25°C under 16-hr photoperiod with an intensity of 4,000 lx (roughly 1,600 μw/cm^2 of irradiance) provided by cool white fluorescent tubes (Sylvania-NEC FL 20 SW/100 V). After 8 weeks of cultivation, shoot-buds produced in potato discs were examined.

Choice of basal media:

The medium formulations used were a modified White's medium described by OKAZAWA *et al.*[10], two modified MS medium[7] by SHEPARD and TOTTEN[11], the other by JARRET *et al.*[3,4] and LAM[5,6], and a medium described by NITSCH and NITSCH[8]. None of original formulation of medium were effective for shoot-but formation, except that a modified medium C and D of SHEPARD and TOTTEN[11] could first produce shoot-buds in extent.

Choice of organic addenda:

Organic components were used as follows : formula of MURASHIGE and SKOOG[7] ; formula of OKAZAWA[4] ; and formula of SHEPARD and TOTTEN[11] modified by NITSCH and NITSCH[8]. Only NITSCH and NITSCH's vitamins and SHEPARD and TOTTEN's organic addenda gave the results of shoot-buds. All media were sterilized by autoclave at 120°C for 10 min.

Results and Discussion

I. Achievement of shoot-bud formation in potato tuber discs *in vitro*

In a preliminary work, freshly excised potato discs had been incubated in SHEPARD and TOTTEN's medium C and D for 90 days with some modification of organic addenda. The results are shown in Table 1. The differentiation of shoot-bud in potato discs has long been known to be possible but difficult to reproduce[10,11] while the initiation of adventitious root was readily established[10] under the conditions of a balance between two growth regulators[12]. Upon the addition of mannitol to the culture under illumination inoculated discs produced green nodules and shoot-buds in some extent, while the nodules change to develop into shoot-bud structure. In this case

none of cultures appeared to differentiated root like structures under the illuminating conditions. The most important component in the culture was the plant growth regulators. Zeatin and indole-3-acetic acid supplement to the medium (ZI medium) was the only effective combination for shoot-bud formation.

Basal medium: Ammonium salt and full strength of mineral elements of MS medium have been reported to exert some detrimental effect on organogenesis in tissue cultures[6]. Decrease in ionic strength of MS medium reversely caused in callus formation, whereas the modified White's medium with relatively a low ionic strength resulted in frequently shoot-bud formation in the culrure. In the present research, the revised formulation by OKAZAWA *et al.*[10] appeared to propose more favorable conditions for the shoot-bud formation. Table 2 shows the effect of basal mineral salts component of medium on the differentiation of potato tissue cultures. The modified White's medium supplemented with NITSCH and NITSCH's vitamins was used for the following experiments.

Vitamins: The levels of the following vitamin formulae had been tested in groups: of MS medium; of OKAZAWA *et al.*[10]; and of NITSCH and NITSCH[8]. The results did not have any significance each other while increase in folic acid level gave rise to breach the cultures under illumination, though the elimination of this factor caused browning of the cultures. The formula of NITSCH and NITSCH has been retained unchanged. The requirements of individual vitamins in the formula was not extensively tested. Biotin and folic acid were added to the medium as the saturated bicarbonate solutions.

TABLE 1. Response of potato discs to light and mannitol

The medium used was Shepard and Totten's medium C and D modified from Lam's medium with or without 45 g/l mannitol. The potato discs (1×6 mm) were cultured for 90 days in 125-ml flasks containing 30 ml medium. The cultures were maintained at 25°C under an illumination of 4,000 lx provided by cool white fluorescent tubes (Sylvania-NEC FL 20 SW/100 V) for 16-hr photoperiod or in continuous dark

Culture conditions		Percentage of differentiation		
		Shoot-buds (%)	Roots (%)	Nodules (%)
Light	+Mannitol	20.6	0	74.7
	−Mannitol	0	0	21.4
Dark	+Mannitol	0	0	68.1
	−Mannitol	0	46.9	84.4

TABLE 2. Kinds of basal medium

The basal media used were mineral salts of MS medium[7], NN medium[8] with or without NH_4^+, and White's medium[10], and organic components of Nitsch and Nitsch[8] with 45 g/l mannitol, 0.5 mg/l zeatin and 0.1 mg/l IAA

Basal medium	Percentage of differentiation		
	Shoot-buds (%)	Nodules (%)	Callus (%)
MS medium	0	29.2	16.7
MS medium $-NH_4NO_3$	16.1	50.0	33.2
1/2 MS medium	16.6	—	—
1/4 MS medium	3.5	—	—
NN medium $-NH_4NO_3$	39.1	66.2	4.2
White's medium	31.0	100.0	92.6

TABLE 3. Effect of adenine, adenosine and adenylate on growth characteristics of potato tuber tissue cultured *in vitro*

Addition 0.2 mM	Percentage of differentiation		
	Shoot-buds (%)	Nodules (%)	Callus (%)
None	25.0	72.2	30.6
Adenine	27.8	77.8	19.4
Adenosine	38.9	80.5	13.9
Adenylate	19.4	94.4	55.5

Nitrogenous compounds: Two types of nitrogenous compounds were known to be important to promote the induction of shoot-bud formation, *i.e.*,-amino acid mixture and adenine. Casein hydrolysate, Casaminoacids Difco Lab., was used extensively. The lesser response of shoot-bud formation on the MS medium was probably due to the relatively high nitrogen content, especially NH_4NO_3. Casaminoacids was not only effective to replace reduced type of nitrogen source but also due to unknown factors of organic and inorganic compounds. The additions were 250 mg/l to 1,000 mg/l of Casaminoacids to the culture medium as final concentration.

The second factor for the shoot-bud formation in potato discs was adenine sulfate which had been known to promote shoot formation in other plant tissue cultures. Table 3 shows the effect of addition of adenine, adenosine and adenylate on shoot-bud formation in potato discs. Addition of adenine sulfate was not as effective as expected while supplement of

adenosine was the best. The amount of adenosine effective for the shoot-bud formation added to the culture medium was 40 mg/l indicated in Table 4.

Carbohydrate: One of the critical factors for the shoot-bud formation in potato discs was the level of sugars utilized as carbon and energy source. Even though potato tissue stores a large amount of reserve starch and potato tissue cultures are capable of utilizing sucrose and glucose to extent as energy sources[4], the addition of sucrose to the culture medium profoundly influenced the growth characteristics of potato discs. At sucrose concentration of 2% potato discs grew for 2 to 3 weeks to form green compact callus which became brown color and finally died. While sucrose concentrations in medium was reduced to 0.1%, potato discs became pale green in surface and initiated the shoot-buds and green nodules. In a series of comparative test 1.0% dextrose was definitely better than 0.5% sucrose, and 0.5% dextrose was often somewhat less effective than no carbohydrate support in culture

TABLE 4. Amount of adenosine effective for shoot-bud formation added to the culture medium

Adenosine Concentration	Percentage of differentiation		
	Shoot-buds (%)	Nodules (%)	Callus (%)
None	9.4	59.4	31.3
Adenosine 20 mg/l	18.8	34.4	78.1
Adenosine 40 mg/l	38.8	50.0	21.8
Adenosine 80 mg/l	17.2	59.4	15.6

TABLE 5. Effect of addition of dextrose to medium on shoot-bud and nodule formation in potato discs cultured *in vitro*

The medium used was the modified White basal medium with supplemented Nitsch and Nitsch's vitamins and 45 g/l mannitol, 0.5 mg/l zeatin, 0.1 mg/l IAA, 1 g/l Casamino acids plus 40 mg/l adenosine: designated as ZI medium. The cultures were maintained at 25°C under illumination of 4,000 lx for 16hr photoperiod

Addition	Percentage of differentiation	
	Shoot-buds (%)	Nodules (%)
None	43.8	87.5
Dextrose 0.5%	41.6	86.1
Dextrose 1.0%	19.9	86.1
Dextrose 2.0%	0	79.5

medium for shoot-bud formation in high frequency. These results indicate that sucrose is not favorable for the shoot-bud initiation in potato discs and carbohydrate was supported by self-stored starch in this case. Table 5 shows that even the addition of dextrose to the medium is not favor for the shoot-bud formation in potato tissue cultures.

Mannitol as osmoticum: The importance of osmotic conditions in the cultures of potato leaf protoplasts have been documented[11]. When all other parameters were optimized for shoot formation in tissue cultures[12], the osmotic potentials which were usually found not to influence the development of shoot critically or not to be favorable for the growth of tissue culture assumed to be governed by medium ingredients-*i. e.*, salts and sugars. In an attempt to investigate any possible osmotic involvement in shoot-bud formation in potato discs, mannitol played a significant role for the initiation of shoot-bud formation in a very much critical concentration (Table 6). Equal osmolar concentrations of inositol and sorbitol were more effective than mannitol but our economy could not afford them. $CaCl_2$ was failed (Table 7). Sucrose and dextrose tested could not replace mannitol as osmotic requirement for the shoot-bud formation. Research of BROWN *et al.*[1] has shown that shoot formation in tobacco callus requires carbohydrate for both a carbon-energy supply and as an osmotic agent. The ability of tobacco callus to produce shoots when a third of the sucrose supply is replaced by mannitol supports this conclusion, since mannitol is not metabolised by this plant and could act as an inert osmoticum[1].

Auxin and cytokinin: Organogenesis *in vitro* is usually controlled by the

TABLE 6. Influence of mannital as osmoticum on shoot-bud formation in potato discs cultured *in vitro*

D-mannitol added to ZI medium. The cultures were incubated at 23°C under an illuminated growth chamber of 4 klx for 16-hr photoperiod. Osmotic potenials were measured by determination of freezing point (Tf). $\pi = 12.22$ Tf

Mannitol addition to the medium		Osmotic potential of the medium	Percent differentiation	
			Shoot-bud	Nodules
(%)	(M)	(Bar)	(%)	(%)
0	0	2.032	0	0
1.6	0.08	4.064	0	32.1
2.4	0.13	5.410	15.8	64.3
3.0	0.18	6.604	31.8	89.3
4.0	0.22	7.622	13.3	96.1

Potato tuber tissue was 9.86 bar.

TABLE 7. Kinds of osmoticum on shoot-bud formation

The one fifth molar or equivalent ionic strength adjusted medium

Osmoticum		percentage of shoot-bud formation (%)
$CaCl_2$	0.067 M	0
Sorbitol	0.2 M	44.4
Inositol	0.2 M	37.0
Mannitol	0.2 M	14.8

TABLE 8. Influence of zeatin and IAA on the shoot-bud formation in potato discs cultured *in vitro*

The medium used was the modified White's mineral salts[10], vitamins of Nitsch[8], 1 g/l Casamino acids plus 40 mg/l adenosine, and 45 g/l mannitol. Concentrations of zeatin and IAA were indicated in table. The cultures were incubated at 23°C in an illumination chamber of 4,000 lx for 16 hr photoperiod

Concn. of IAA, mg/l	Concentration of zeatin, mg/l			
	0	0.1	0.5	1.0
0				
0.001			SF 13.3 NF 21.1	
0.01		SF 14.2 Callus	SF 75.6 NF 59.6	SF 85.0 NF 32.7
0.1	Callus	SF 28.6 Callus	SF 83.3 NF 57.7	SF 53.6 NF 51.6
1.0	Friable Callus	Green Callus	Green Callus	Green compact Callus

SF: % of shoot-bud formation in discs.
NF: % of nodule formation in discs.

kind and the concentration of plant growth regulators added to the culture medium[12]. The results of the present investigation in Table 8 shows that the auxin-cytokinin interactions in part provoke the expected morphogenic responses in potato discs. The initiation of shoot-bud formation in potato discs was stimulated by the simultanous application of auxin (0.01–0.1 mg/l IAA) and cytokinin (0.4–1.0 mg/l, zeatin), whereas a marked deterioration of the organogenic activity was observed when subjecting them to the higher

concentration of auxin (1.0 mg/l or more of IAA). As a matter of fact that zeatin and IAA was the only combination tested effective to induce shoot-bud formation in potato discs, including 2, 4-D, NAA, benzyladenine, isopentenyladenine, and kinetin. Amongst the rest of combination, benzyladenine-IAA and isopentenyladenine-IAA were effective to certain extent for shoot-bud formation in potato discs, which produced shoots upon transferring to a proper medium supplemented with zeatin-auxin and dextrose of proper concentrations. Kinetin was completely inert for shoot-bud formation shown in Table 9.

pH of medium: The pH of the medium was adjusted with a few drops of 1 M HCl or with NaOH before the agar was added to the medium which had been heated 10 min in the autoclave. The pH of the medium tends to drift toward either more acidic or basic conditions during heating for sterilization when the pH of the medium varied in the range of 5.0 to 7.0. One of Good's buffer, MES (2-N-morpholino-ethanesulfonic acid) was used in 5 mM to correct the pH value for this purpose. Table 10. indicates the

TABLE 9. Influence of kind of cytokinin on shoot-bud formation in potato discs cultured *in vitro*

Discs were first inoculated to each kind of cytokinin-IAA media for 8 weeks then transferred to zeatin-IAA medium and cultured for another 4 weeks. 0.5 mg/l cytokinin and 0.1 mg/l IAA were added to the medium

	Percentage of shoot-bud formation			
	Zeatin (%)	Benzyl-adenine (%)	Isopentenyl-adenine (%)	Kinetin (%)
1st explant	40.8	0	0	0
2nd transfer	100.0	55.9	50.0	0

TABLE 10. Influence of pH of the medium on the shoot-bud formation in potato discs

All the parameters of the cultures were optimized. The pH of the medium was adjusted to indicated values below with 1 M NaOH solution. MES, 2-(N-morpholino)-ethanesulfonic acid, 5 mM was used as buffer solute

Culture condition (pH)	Percentage of shoot-bud formation (%)
5.4	60.3
5.8	71.0
6.2	98.2
7.0	27.3

values between 5.8–6.0 gave the best score for shoot-bud formation.

Composition of the defined medium for shoot-bud formation: The amount of medium ingredients finally adopted for the medium for shoot-bud formation in potato tissue cultures are listed in Table 11. designated as ZIG medium. Since gibberellic acid, GA., was reported to be effective on potato shoot-bud formation, the addition of 0.1 mg/l of GA was also listed in this medium formula while the effect of GA on shoot-bud formation were not clear in extent of the physiological conditions of potato tubers used as experimental materials[9]. GA was believed to decompose 90% of the activity during heating at 120°C, and still gave sometimes good results.

TABLE 11. Composition of medium for shoot-bud formation in potato tuber tissue cultured *in vitro*

The pH of the medium have to be adjusted to 6.0 with a few drops of 1 M NaOH befor the addition of agar and the heating in autoclave at 120°C for 10 min. The cultures are maintained at 20°C under an illumination of 4 klx provided by cool white fluorescent tubes for 16 hr photoperiod

Inorganic components (mg/l)		Organic components (mg/l)	
$MgSO_4 \cdot 7H_2O$	756	Inositol	100
$Ca(NO_3)_2 \cdot 4H_2O$	288.7	Adenosine	40
Na_2SO_4	200	Nicotinic acid	5.0
KNO_3	80	Glycine	2.0
KCl	65	Thiamine-HCl	0.5
$NaH_2PO_4 \cdot 2H_2O$	21.4	Pyridoxine-HCl	0.5
$MnSO_4 \cdot 4\text{-}6H_2O$	6.5	Folic acid	0.5
$ZnSO_4 \cdot 7H_2O$	3.75	Biotin	0.05
H_3BO_3	1.5	IAA	0.1
KI	0.7	GA	0.1
Na_2MoO_4	00.3	Zeatin	0.5
$CoCl_2$	0.03	Casamino acids	1000
$CuSO_4$	0.02	Sucrose	1000
Fe-EDTA	38.2	MES	1000
		Mannitol	45000
pH adjusted to 6.0		Agar Bacto	8000

IAA: Indole-3-acetic acid;
GA: Gibberellic acid;
Casamino acids: Casein hydrolysate, Bacto certified grade of Difco Lab.;
MES: 2-(N-Morpholino)-ethanesulfonic acid.

II. Explants, discs excised from potato tubers.

Size: An explant is a piece of tissue which is isolated from the plant for the materials of culture. The physiological conditions of explant influence the type and extent of morphogenesis. As a general rule, very small explants have low survival rate in culture. OKAZAWA *et al.*[10] found the spontaneous formation of shoot-buds in the relatively large explant without auxin-kinetin. No shoot-bud was successfully produced from small size explant. Although potato tubers were known to contain gibberellic acid endogenously the level of which progressively increased by the time of sprouting[9], the ability of small size potato discs to produce shoot-buds when all parameters other than the size of discs were optimized lends support the morphogenetic potentials distributed even in a small size discs (Table 12). JARRET *et al.*[3] reported that an addition of exogenous gibberellic acid to the medium was essential for the initiation of shoot-bud formation in potato discs. In general potato

TABLE 12. Influence of size of potato discs on the ability of shoot-bud formation

The cultures were incubated in the optimal conditions described in Table 11. except zeatin, IAA and GA listed

Medium	Percentage of differentation			
	Small size discs (1×3 mm diameter)		Standard size discs (1×6 mm diameter)	
	Callus formation (%)	Shoot-bud formation (%)	Callus formation (%)	Shoot-bud formation (%)
Z G	8.3	0	55.9	0
Z I	59.3	12.2	100	12.5
Z I G	100	22.2	100	38.5

TABLE 13. Influence of numbers of potato discs inoculated in a flask for shoot-bud formation

Numbers of discs in flask	% of shoot-bud formation (%)	No. of Shoot-bud	
		in flask	in disc
2	50.0	1.5	1.5
3	17.8	1.0	2.0
4	87.5	5.5	1.6
5	90.0	9.5	2.1
6	83.3	8.5	1.7
9	100	15.5	1.7

discs, excluding meristems or vascular bundles, young tubers have a high degree of morphogenic competence than older tissue.

Numbers: Influence of the numbers of potato discs cultured in a flask containing 30 ml of ZIG medium was checked. There were not any distinguishable influences observed in cultures (Table 13). The optimal number is designed for 5, because more than five increases a contamination chance during inoculation work.

III. Enviromental conditions.

Temperature: Light and temperature were also critical factors in potato shoot-bud formation. The optimum temperature for shoot-bud formation was 20±2°C and the frequency of the shoot-bud formation decreased at higher or lower temperature (Table 14).

Light: Light intensity appeared to have a pronounced influence on the shoot-bud formation in potato discs. The shoot-buds were almost completely inhibited under less than 1,000 lx of light (Table 15), while the minimum temperature for the shoot-bud formation in potato was 16°C with 2,000 lx of light intensity. At high light intensity the temperature inside the culture

TABLE 14. Influence of temperature on the shoot-bud formation in potato discs cultured *in vitro*

The cultures were maintained in the optimal conditions described in Table 11. except incubation temperatures

Incubation temperature (°C)	Percentage of shoot-bud formation	
	ZI medium (%)	ZIG medium (%)
16	0	18.8
21	39.3	82.1
26	21.4	37.5
21	18.8	25.0

TABLE 15. Dependency of light intensity on the shoot-bud formation in potato discs cultured *in vitro*

Light intensity (lx)	Percentage of shoot-bud formation	
	ZI medium (%)	ZIG medium (%)
0	0	2.3
1,000	16.6	25.0
2,000	41.1	82.1
4,000	45.6	89.0

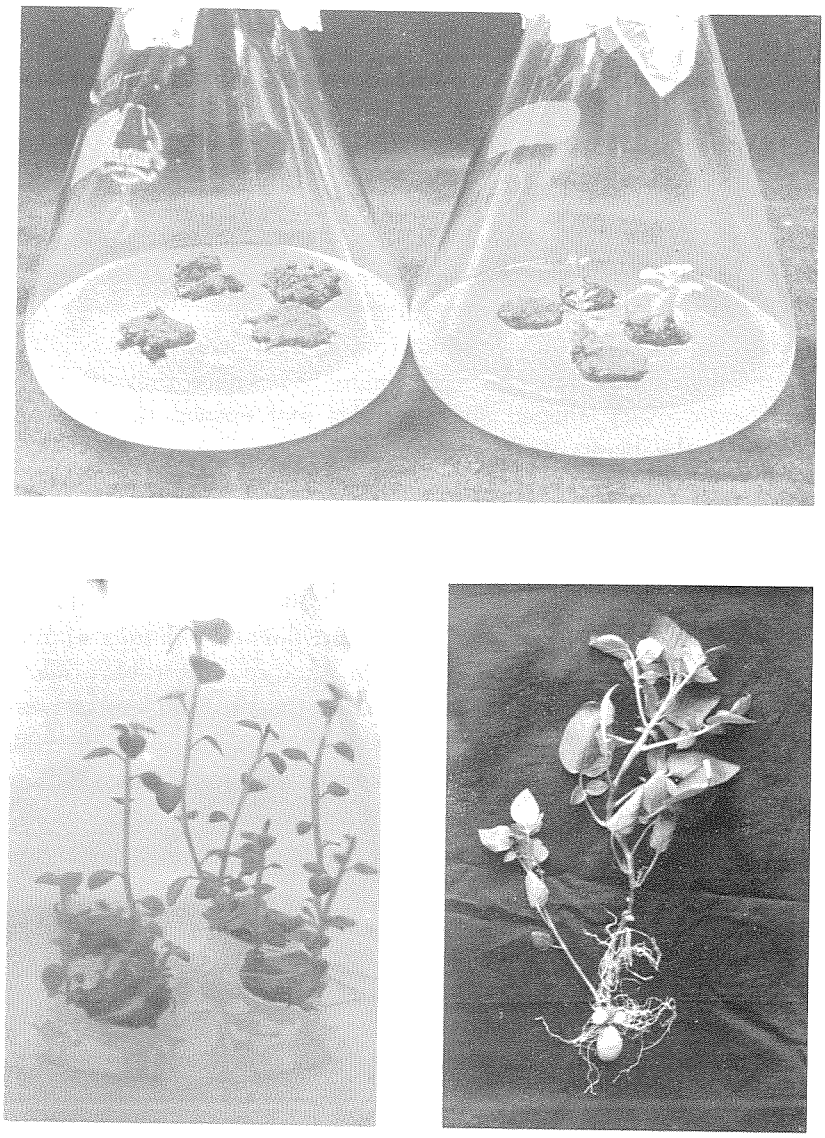

Fig. 1. Formation of shoot-buds followed by plant regeneration from potato tuber tissue cultured *in vitro*.

Top: Nodule formation (left) followed by shoot-bud formation in potato discs.

Left: Small ovary shaped simple leaves and lateral roots developed on the newly formed shoots in potato discs.

Right: Regenerated plant with small tubers from the shoot initiated in potato discs.

flasks increases 2-3°C above that of the growth chambers. JARRET *et al.*[2] reported that the extent of the morphogenic responses due to the photon flux density was very dependent on temperature with the lower the temperature being most benefitial.

IV .Plant regeneration.

For regeneration of plantlet, the defined medium for shoot-bud formation presented here gave satisfactory results. On this case the lateral roots were adventitiously produced from the newly formed shoots while culturing in this medium without transferring to new medium.

Regenerated plantlet with a shoot-root system were successfully established in moistened jiffy-mix and vermiculite bed where they were possible to develop some ovary shaped simple leaves and ensued to produce some small tubers as the plants grew longer (Fig. 1).

Conclusion

The critical factors which determine morphogenesis of potato tuber discs to shoot-buds are the kinds of plant growth substances, the osmotic potentials of the culture medium and the nutritional conditions of nitrogen-carbohydrate balance in the medium. On this conection of evidence, an additions of gibberellic acid or abscisic acid to the medium and a manipulation of light intensity and temperature also given conditions necessary for active cell division and differentiation to meristematic nodules followed by shoot-bud formation.

Summary

Discs (1×6 mm, diameter) excised from tubers of potato (*Solanum tuberosum* L. cv. Irish Cobbler) were induced to differentiate *in vitro*, producing shoot-buds or callus cultures in an illuminated growth chamber.

The basal nutrient medium consisted of inorganic salts according to OKAZAWA *et al.*[10], vitamins according to NITSCH and NITSCH[8], casamino acids, adenosine, mannitol, and agar. Whilst carbohydrate was supported by self-stored starch.

Zeatin and indole-3-acetic acid added to the medium induced shoot-buds in potato discs, but the other cytokinins tested did not. Gibberellic acid was effective for shoot-bud induction when discs were excised from the freshly harvested tubers.

Plantlets were readily regenerated in the same medium, upon transferring to jiffy-mix and vermiculite bed where they were possible to produce small

tubers as plants grew longer.

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