Influences of Environmental, Hormonal and Nutritional Factors on Potato Tuberization *in vitro**

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The process of potato tuberization is controlled by environmental conditions; short days and low night temperatures (inducing conditions) stimulate the process, whereas long days and high temperatures (non-inducing conditions) retard the advance of the process^{3,4,10,31}). Several workers suggested the occurrence of a specific tuber forming stimulus which is formed in the potato leaves under the inducing conditions and transmitted into stolon tips to induce the tuberization^{4,10,18}). On the other hand, a number of investigations indicated that endogenous plant hormones would also participate in the process. It has been revealed that the tuberization is closely correlated with a decrease in gibberellin (GA) levels and high GA levels inhibit the process^{23,24,29,30}). However, the roles of cytokinins and abscisic acid (ABA) are still a matter of controversy. For example, an application of a cytokinin to culture medium induced the tuberization *in vitro*^{19,21,26}), whereas that to intact plants failed to induce tubers and caused loss of diageotropic growth habit of the stolons³⁴). With regard to the role of ABA on the tuberization, several workers reported a promotive effect^{1,8}), however others made an objection to the effect^{5,27,32}).

In the present investigation, we examined effects of environmental, hormonal and nutritional factors on the tuberization by means of stem segment culture *in vitro*, to know the causal relationship between these hor-

mones and potato tuberization.

Materials and Methods

Plant materials

Freshly harvested potato tubers (*Solanum tuberosum* L. cv. Irish Cobbler) were stored at 4°C in the dark for at least 2 months until the end of their endogenous dormant period. Unless otherwise specified, the tubers used were relatively young ones which had been stored for within 6 months. Sprouting tubers were obtained by placing them at 25°C in the dark for 3 weeks. The tissue plugs (20 mm in diameter and 25 mm in thickness) bearing a single sprout of 1 cm long were excised by a cork borer and surface sterilized with 1% sodium hypochlorite solution for 10 min. The plugs were washed thoroughly with running tap water and then a few drops of 0.1 M calcium nitrate were applied on to the top of the sprout to prevent necrosis of the apex⁷). Subsequently, they were kept in a moist chamber at 25°C for 3 days to form wound periderm and then surface sterilized again. These were planted in a plastic pot containing vermiculite moistened with HOAGLAND nutrient solution¹²). Etiolated shoots of about 30 cm long were obtained by keeping the pots at 25°C in the dark for 3 weeks. The shoots were harvested to prepare single-node segments which were obtained between the second and seventh node. These segments were sterilized with 1% sodium hypochlorite solution for 1 h, washed 5 times with sterile water and finally each 5 mm end of the segment was cut off. The remaining single-node segments were used as inocula for tissue culture.

Tissue culture

Three pieces of the single-node segments

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were explanted in a 100-ml Erlenmeyer flask containing 20 ml of the revised WHITE's medium supplemented with various addenda. Five replications were prepared for each treatment. Unless otherwise stated, the organic constituents in 1 l basal medium were as follows: 20 g sucrose, 6 g Bacto-agar, 1 g casamino acids, 100 mg inositol, 5 mg thiamine-HCl, 2 mg pyridoxine-HCl, 5 mg Ca-pantothenate and 1 mg nicotinic acid. The formula of inorganic constituents was the same as described by OKAZAWA *et al.*²⁵⁾ except extra supplement of 10 mM CaCl_2 . The cultures were maintained at 25°C in the dark for 3 weeks. At the end of each culture period, the rate of tuberization was calculated as (the number of tuberized laterals)/(the total number of emerged laterals). The criterion for tuberization was either an occurrence of a visible swelling at the sub-apical region of the laterals or an occurrence of incipient tuber.

Extraction and bioassay for cytokinin

In order to determine the levels of endogenous plant hormones in the single-node segments, the samples were homogenized with a sufficient amount of ethanol to give a final 70% ethanolic extract, kept overnight at 4°C and then filtered. The subsequent procedure for cytokinin extraction was essentially the same as described previously¹⁶⁾. Unlike the previous experiment, however, the eluate from cation exchange column was directly subjected to paper chromatography developed with isopropanol:ammonia:water (10:1:1 v/v). Cytokinin activity was determined using soybean callus bioassay¹⁴⁾.

Extraction and bioassay for ABA-like substance

The ethanol extract was evaporated to remove ethanol, adjusted to pH 2.5 with 1 N HCl and then partitioned 3 times against equal volumes of ethyl acetate. After removal of the aqueous phase, the remaining ethyl acetate phase was partitioned 3 times against a half volumes of 0.5 M phosphate buffer (pH 8.0). Subsequently, the aqueous phase was acidified to pH 2.5 and extracted with 3 equal volumes of ethyl acetate. The combined ethyl

acetate extracts were dried over sodium sulfate anhydride, filtered and then evaporated to dryness. The resulting residue was subjected to paper chromatography developed with the same solvent as stated above. Activities of ABA-like substance were detected by the inhibition of *Avena* coleoptile straight growth induced by 10^{-7} M indole-3-acetic acid.

Extraction and bioassay for GA-like substance

The procedure of extraction for GA-like substance was the same as the case of ABA-like substance other than using 1 M phosphate buffer (pH 6.2). GA-like activities were determined by the amount of reducing sugar liberated from the incubated embryoless endosperms of *Avena* seeds¹⁵⁾.

Results

Effect of temperature

Effect of temperature on *in vitro* tuberization was examined at different sucrose concentrations. As shown in Fig. 1, the tuberization was scarcely observed at 30°C at any concentrations of sucrose, whereas tubers were spontaneously formed with a high rate when cultured at 10°C. The rate of tuberization at 25°C was improved with raising the sucrose concentrations. Hereafter, all experiments were performed exclusively at 25°C.

Fig. 1. Effect of the culture temperature on the *in vitro* tuberization at different sucrose concentrations.

Effect of zeatin riboside (ZR)

According to the evidence that a major cytokinin naturally occurring in potato tubers is ZR^{13,16,19)}, the present experiment was designed to assess the availability of this substance for stimulating tuberization (Fig. 2). When the segments were cultured in combination with 2% sucrose, ZR exerted no stimulating effect on the tuberization at any of the concentrations. Even though the concentration raised to 10^{-5} M, no tuberization was observed, this substance stimulating only branching of the laterals (data was not presented). However, ZR exhibited a promoting effect on the tuberization at sucrose concentrations above 4%. But the effect was only cumulative to the effect of sucrose. The fresh weight of the tubers formed was increased by addition of ZR. In combination with 4% sucrose, the fresh weights at concentrations of 0, 10^{-7} and 10^{-6} M ZR were 49.3 ± 17.9 , 67.0 ± 23.6 and 71.1 ± 25.9 mg ($\pm$ SD, $n=10$), respectively.

Effect of ABA

In order to examine whether or not ABA plays a causal role in the tuberization, the cultures were performed at various concentrations of ABA in combination with 2% sucrose. As 2% sucrose by itself exhibited no promoting effect on the tuberization as mentioned above, this experiment would be suited for evaluation of the availability of ABA for the tuberization. As shown in Fig. 3, the rate of tuberization appears to increase progressively with raising the ABA concentration. However, the feature of all formed tubers was characterized by only slight swelling at the sub-apical region of the laterals, and no further development proceeded to typical spherical shape with dormant buds was observed.

Interactive effect of gibberellic acid (GA_3) and ABA

Effects of GA_3 and ABA were examined in combination with 8% sucrose. The rate of tuberization in the absence of both GA_3 and ABA was 0.8 (Fig. 4). With raising the ABA concentrations, the rate slightly decreased. An application of 10^{-7} M GA_3 considerably inhibited the tuberization, whereas simultaneous application of ABA

Fig. 2. Effect of ZR on the *in vitro* tuberization at different sucrose concentrations.

Fig. 3. Effect of ABA on the *in vitro* tuberization. Sucrose concentration in the medium was 2%.

recovered the inhibition. Such recovery was not observed when the concentration of GA_3 was raised to 10^{-6} M.

Effects of (2-chloroethyl) phosphonic acid (CEPA) and 1-aminocyclopropane-1-carboxylic acid (ACC)

Effects of CEPA which is an effective ethylene releasing reagent and ACC which is a precursor of ethylene²⁾ are shown in Fig. 5. Applications of these compounds caused diageotropic growth habit of the laterals, but failed to induce the tuberization at any of the concentrations.

Fig. 4. Interactive effect of GA_3 and ABA on the *in vitro* tuberization. Sucrose concentration in the medium was 8%.

Fig. 5. Representative appearances of (2-chloroethyl) phosphonic acid (CEPA) and 1-aminocyclopropane-1-carboxylic acid (ACC) treated cultures after 3 weeks. Sucrose concentration in the medium was 2%.

Effect of age of the tubers used to obtain the single-node segments

The experimental results described thus far were obtained by using the single-node segments derived from relatively young tubers (stored for within 6 months). As mentioned above, these segments scarcely form tubers in combination with 2% sucrose. However, when the single-node segments derived from old tubers were cultured in this condition, spontaneous tuberization occurred, and the rate of the tuberization increased with advance of the age of the tubers (Fig. 6). When the segments derived

Fig. 6. Changes in the tuberizing ability of the single-node segments with the advance of the age of the tubers used to obtain them. Sucrose concentration in the medium was 2%.

from new and senile tubers (stored for 4 and 14 months respectively) were cultured, a gross difference in the rate of tuberization was observed which is shown in Fig. 7. If endogenous plant hormones were really responsible for the tuberization, the levels of the hormones in these two kinds of inoculum should be different. Therefore, comparisons were made between the levels of the hormones; GA-like substance, cytokinins and ABA-like substance, in these two kinds of inoculum. As shown in Fig. 8, the single-node segments obtained from the young tubers exceeded in GA activity as compared with that from the senile ones. Cytokinin activities were detected as two components migrated at R_f 0–0.2 and 0.4–0.7 on the paper chromatograms (Fig. 9). The slow-moving activity seems to correspond to zeatin ribotide, and the fast-moving one to zeatin, its glucoside and riboside¹⁶). While the level of the former was prominent in the segments derived from the young tubers, the level of the latter was not different. As to the level of ABA-like substance, there was no appreciable difference between the segments derived from the different aged tubers (Fig. 10).

Effect of nitrogen

Effects of various nitrogen sources on the

Fig. 7. Appearances of cultured single-node segments derived from young (A) and senile (B) tubers after culturing for 3 weeks. Sucrose concentration in the medium was 2%. Vertical bars indicate the length of 1 cm.

Fig. 8. Comparison of endogenous GA activities in the single-node segments derived from young (A) and senile (B) tubers. Each extract, equivalent to 20 g fresh weight, was chromatographed on paper and assayed using *Avena* endosperm test. Broken lines represent the amount of reducing sugar liberated from the endosperms by 10^{-6} and 10^{-7} M GA_3 .

Fig. 9. Comparison of endogenous cytokinin activities in the single-node segments derived from young (A) and senile (B) tubers. Each extract, equivalent to 15 g fresh weight, was chromatographed on paper and assayed using soybean callus. Broken lines represent callus yields with 30 and 300 ng/l zeatin.

tuberization were examined using single-node segments derived from 9-month-old tubers. The basal medium employed here was free from casamino acids. The medium itself contains 3.2 mM nitrate as nitrogen source. The rate of tuberization on the basal medium was between 0.60 and 0.76. Enrichment of nitrate exerted no appreciable effect on the tuberization, whereas that of ammonium and casamino acids produced, to almost equal extent, a detrimental effect on the tuberization keeping pace with raising their concentrations (Fig. 11). Effects of sucrose and ammonium nitrate are shown in Fig. 12. Ammonium nitrate markedly inhibited the tuberization in combination with 2% sucrose. However, raising the sucrose concentration above 4% negated the effect of ammonium nitrate on the tuberization.

Discussion

Although several possible bases for the tuberization in potato plants have been of-

Fig. 10. Comparison of endogenous ABA activities in the single-node segments derived from young (A) and senile (B) tubers. Each extract, equivalent to 10 g fresh weight, was chromatographed on paper. The activities were measured by inhibition of *Avena* coleoptile straight growth. Broken lines represent the percent of control growth obtained by 10^{-8} and 10^{-7} M ABA.

Fig. 11. Effects of various nitrogen sources on the *in vitro* tuberization. The single-node segments derived from old tubers were used as inoculum, and sucrose concentration in the medium was 2%. The casamino acids contain 10% nitrogen.

Fig. 12. Effects of ammonium nitrate on the *in vitro* tuberization at different sucrose concentrations. The single-node segments derived from old tubers were used as inoculum.

ferred, the precise cause resulting in the tuberization remains to be still inconclusive. WERNER³³), in his classical works, proposed that carbohydrate/nitrogen ratio is the causal factor in the tuberization. However, recently a number of studies cast doubt on his concept. In the present experiment, *in vitro* tuberization appears to have a close association between culture temperature and sucrose concentration in the medium, viz. the rate of tuberization was proportional to increase in the sucrose concentration when cultured at 25°C, whereas the tuberization was scarcely observed at 30°C at any of the sucrose concentrations (Fig. 1). In addition to the above fact, detrimental effect of nitrogen on the tuberization was not observed when cultured with above 4% sucrose (Fig. 12). These results contradict WERNER's opinion but support a previous finding, in which the tubers were formed spontaneously in the presence of 8% sucrose but not 2% sucrose²²). It seems more feasible that some naturally occurring hormonal substance would be implicated in the induction of the tuberization as mentioned by many workers^{4,10}).

In this context, a possible involvement of a cytokinin in the tuberization has been

proposed by PALMER and SMITH²⁶⁾, who reported induction of *in vitro* tuberization by an application of a cytokinin, and this assumption was supported by several workers^{19,21)}. These earlier experiments were carried out in combination with a high concentration of sucrose (6%). In the present experiment, however, ZR exhibited no stimulating effect on the tuberization at a low sucrose concentration (2%) (Fig. 2). Furthermore, the cytokinin level in the stem segments which readily form tubers was not different from that in the segments which hardly form tubers (Fig. 9). These results seem to indicate that cytokinin is not the direct cause for the tuberization. In view of the fact that ZR increased the fresh weight of tubers formed in combination with high sucrose concentrations (above 4%), cytokinins may only stimulate thickening growth involving cell division to form tubers.

An application of ABA induced swelling at the sub-apical region of the laterals but failed to induce incipient tubers with dormant buds (Fig. 3). Additionally, the level of ABA-like substance in the stem segments which readily form tubers was not significantly different from that in the segments which hardly form tubers (Fig. 10). These results suggest that ABA is incapable of inducing the tuberization, inconsistent with the results by EL-ANTABLY *et al.*⁸⁾.

GRACIA-TORRES and GOMEZ-CAMPO⁹⁾ reported that an application of CEPA led to an increase in the frequency of the tuberization, and suggested the important role of ethylene in the tuberization. But thereafter, several workers^{6,20,28)} have counterclaimed against their opinion. In the present experiment, applications of CEPA and ACC failed to induce the tuberization (Fig. 5). However, these compounds caused diageotropic growth habit of the laterals. In accordance with the view proposed by PALMER and BAKER²⁸⁾, these results indicate that ethylene may play an important role in maintenance of diageotropic stolons.

It has previously been reported that the endogenous GA in potato plants seems to

play an important role in controlling the tuberization and concluded that a reduction of GA level is necessary for inducing the tuberization^{23,24)}. In the present experiment, there found a close correlation between the level of GA and the tuberization, viz. applications of GA₃ strongly inhibited the tuberization *in vitro* (Fig. 4) and the level of GA-like substance was higher in the single-node segments which hardly form tubers than that in the segments which readily form tubers (Fig. 8). However, how the low level of GA is associated with the tuber initiation is unclear at present. A tempting explanation was proposed by several workers^{11,17)} that the tuberization is induced by a direct balance between inhibiting hormones such as GA and promoting hormone such as ABA. But as mentioned above, ABA seems to be incapable of inducing the tuberization. The question what substance promote the tuberization still remains open.

Possible role of some unknown factors such as tuber forming stimulus which was proposed by GREGORY¹⁰⁾ and CHAPMAN⁴⁾ should be examined in detail, especially from physiological and biochemical point of view.

Summary

By means of tissue culture method in which single-node segments obtained from etiolated potato shoots were used as inocula, effects of environmental, nutritional and hormonal factors on *in vitro* potato tuberization were examined. The results obtained here are summarized as follows:

- (1) The rate of tuberization was wholly dependent on the sucrose concentration in the medium when cultured at 25°C, but was unassociated with it if cultured at either 10°C or 30°C.
- (2) Cytokinin (ZR) produced no stimulative effect on the tuberization in combination with 2% sucrose, even though its concentration raised up to 10⁻⁵ M. However, this substance induced an increase in fresh weight of the tubers formed in combination with high sucrose concentrations (above 4%).

(3) ABA increased the rate of the tuberization with raising its concentration in combination with 2% sucrose, but the formed tubers characteristically ceased their development at swelling stage.

(4) GA₃ markedly inhibited the tuberization.

(5) Besides a modification of lateral shoot orientation, CEPA and ACC caused no stimulation of the tuberization.

(6) The single-node segments derived from senile tubers produced more rapidly new tubers than those from young ones. There was no significant difference in the levels of endogenous cytokinins and ABA-like substance between the single-node segments derived from young and senile tubers, whereas the former exceeded in the level of GA-like substance as compared with the latter.

(7) Applications of compounds containing reduced nitrogen inhibit the tuberization, while nitrate exerted less inhibitory effect on the tuberization. The inhibitory effect of reduced nitrogen was only observed at a low sucrose concentration (2%).

These results described above seem to indicate that a reduction of GA level is prerequisite for the tuberization and that neither cytokinin nor ABA is responsible for the tuberization. Cytokinin may stimulate growth of the tubers, and ABA seems to play a supplemental role in the tuberization.

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〔和 文 摘 要〕

組織培養系のバレイシヨ塊茎形成におよぼす諸要因

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黄化徒長茎断片を用いた組織培養法により、バレイシヨ塊茎形成に影響する諸要因について検討を加えた。

1. 組織培養系における塊茎形成は培養温度と培地中の蔗糖濃度により大きな影響を受けた。25℃では、蔗糖濃度の増加に応じて塊茎形成率は増加したが、30℃では、ほとんど塊茎形成は認められなかった。また、10℃では蔗糖濃度にかかわらず、塊茎が形成された。
2. サイトカイニン（ゼアチンリボシド）は低濃度蔗糖（2%）下では全く塊茎形成を引き起こさなかったが、高濃度蔗糖（4%以上）下では塊茎形成促進作用を示した。また形成された塊茎の生重量は、ゼアチンリボシド添加により増大した。
3. アブジン酸は側芽先端部のわずかな肥大を誘起したが、休眠芽を有する塊茎の形成は引き起こさなかった。
4. ジベレリン酸は塊茎形成を強く阻害した。
5. CEPA 及び ACC は側芽の横地性を誘起したが、塊茎形成は全く促進しなかった。
6. 塊茎形成率は黄化徒長茎を得るために用いた親いもの齢の増加に伴い増加した。貯蔵4か月程の比較的若い親いものより得た茎断片の塊茎形成率はほぼ0であったが、同14か月の老化塊茎より得た茎断片のそれは1.0に達した。これらの両茎断片中の内生長物質活性について比較した結果、サイトカイニン及びアブジン酸の活性には大きな差異は認められなかった。ジベレリン活性は老化塊茎より得た塊茎化の容易な茎断片のほうが明らかに低かった。
7. 硝酸態窒素は塊茎形成に影響をおよぼさなかったが、アンモニア態窒素は塊茎形成を強く阻害した。硝酸アンモニウムによる塊茎形成阻害作用は、低濃度蔗糖（2%）下でのみ認められ、高濃度蔗糖（4%以上）下では、その作用は全くみられなかった。
8. 以上の結果は、ジベレリンの減少が塊茎形成の必要条件であり、サイトカイニン及びアブジン酸は塊茎形成の直接的な要因ではないことを示しているものと推察される。サイトカイニンは形成された塊茎の生育を促進し、アブジン酸は塊茎形成に対し、何らかの補助的な役割を担っているものと考えられる。