The action mechanism of cytokinin in breaking dormancy of gladiolus corm

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Summary : As reported in our previous papers, cytokinin is effective in breaking dormancy of gladiolus corms, and hence its action mechanism was investigated in the present study.

Changes in the level of endogenous cytokinin activity in the corms in the releasing process from dormancy caused by low temperature was examined. The activity was hardly detectable in the corms immediately after low temperature storage for 5 weeks, in spite that this treatment was sufficiently effective on breaking dormancy. When the corms were further stored at high temperature for 10 days, however, cytokinin activity in the corms rapidly increased. It seems possible to postulate from these results, that the process of cytokinin synthesis in the corms is prepared by exposure to low temperature and the start of the actual cytokinin synthesis is triggered by high temperature following exposure to low temperature.

In the experiment of the *in vitro* culture of the buds isolated from the corms stored under various conditions, cytokinin synthesis was recognized after low temperature treatment followed by high temperature. The size of explants was related to the ability to sprout, so that it seems to have certain bearing on the process of cytokinin synthesis.

In the experiment using ^{14}C-BA, inhibitors (abscisic acid, pyrogallol and coumarin) which antagonize cytokinin on sprouting of the corms, suppressed the incorporation of cytokinin into RNA. These results indicate that the antagonism between cytokinin and inhibitors in breaking dormancy is related to RNA metabolism.

The summer gladiolus forms new corms on the mother corm from summer through early autumn. Following harvest, corms are dormant for 2 to 3 months. This dormancy disappears after exposing them to a low temperature.

According to the early studies of Denny (1930, 1933, 1935, 1936), who dealt with dormancy of gladiolus corms in detail, the dormant period of freshly-harvested corms could be shortened by the treatment with low temperature from 3°C to 10°C, for 4 to 6 weeks depending on cultivars, while high temperature at 30°C or 35°C did not affect the dormancy.

As described in our previous papers (1972a, 1973), cytokinins were effective in breaking the dormancy of gladiolus corms. How is the dormancy of the corm broken by cytokinins or how do cytokinins work in breaking the dormancy ?

Tsukamoto et al. (1956, 1959, 1972b) reported that dormant corms of gladiolus contained large amounts of acidic growth inhibiting substances, including abscisic acid, and that these substances disappeared from the corms which had come out of their dormant period through

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low temperature treatments. Furthermore, Imanishi (1974) could induce the dormancy of gladiolus cormels with abscisic acid.

Although acidic growth inhibitors in the corm treated with cytokinin did not decrease, the inhibition of sprouting of the corm through abscisic acid was removed by cytokinin, and there was an antagonistic relation between abscisic acid and cytokinin in sprouting (Yazawa: 1975).

Standen et al. (1972a, b) and Hewett et al. (1973), however, reported that in several species of plants, the endogenous cytokinin increased with breaking of the dormancy. As for the molecular effects of cytokinin and inhibitors on regulating the dormancy, van Overbeek (1967) indicated that induction of dormancy in *Lemna minor* by abscisic acid was resulted from inhibited synthesis of nucleic acids and induction of the germination by cytokinin was resulted from more restored synthesis of DNA than that of RNA. Khan (1969), on the other hand, reported that in excised pear embryo inhibition of the incorporation of labelled precursor by inhibitor into ribosomal RNA was removed by cytokinin.

Considering these arguments on the dormancy, experiments were conducted on the following points, aiming at clarifying the action mechanism of cytokinin in breaking dormancy of gladiolus corms.

1; Effect of low temperature storage on the level of endogenous cytokinin in corms.

2; Locus of cytokinin production in corms following low temperature storage for breaking dormancy.

3; Effect of inhibitors [abscisic acid, coumarin and pyrogallol, which inhibited sprouting of gladiolus cormels in our previous experiment (1974)] on the incorporation of ^{14}C-labelled cytokinin into various substances in corms.

Materials and Methods

1 ; *Effect of low temperature storage on the level of endogenous cytokinin in gladiolus corm.*

Corms of cultivar 'Hector' were planted in the Experimental Farm, Kyoto University late in April, 1973. Its flowering season was from mid June to early July. The corms were harvested on September 25 and immediately allowed to dry at room temperature for 2 weeks. Then the corms were divided into 3 lots: the first was stored at 20°C for 5 weeks, the second at 0°C for 5 weeks, and the third at 0°C for 5 weeks and then at 30°C for 10 days, under a dry condition.

Plugs of corm tissues each carrying an apical bud were excised with a cork borer (1 cm in diameter). Each plug was 0.8—1.0 g in weight and 200 g of plugs from each lot was extracted with 1,200 ml of 90% methanol at 20°C for 48 hours. Two hundred milliliter of this extract was filtered, and the residue was washed with 40 ml of 90% methanol. The filtrate and washing were evaporated *in vacuo* at 45°—50°C to an aqueous phase. The fractionation of cytokinin conformed to the method of Standen and Wareing (1972) and Fraction A and B were obtained (Fig. 1).

The Fractions A and B dissolved in 6 ml of 90% methanol were respectively streaked on 2 sheets of Toyoroshi No. 51 chromatography paper (40×40 cm), and separated with *n*-butanol saturated with water. The dried chromatograms were divided into 10 equal strips,

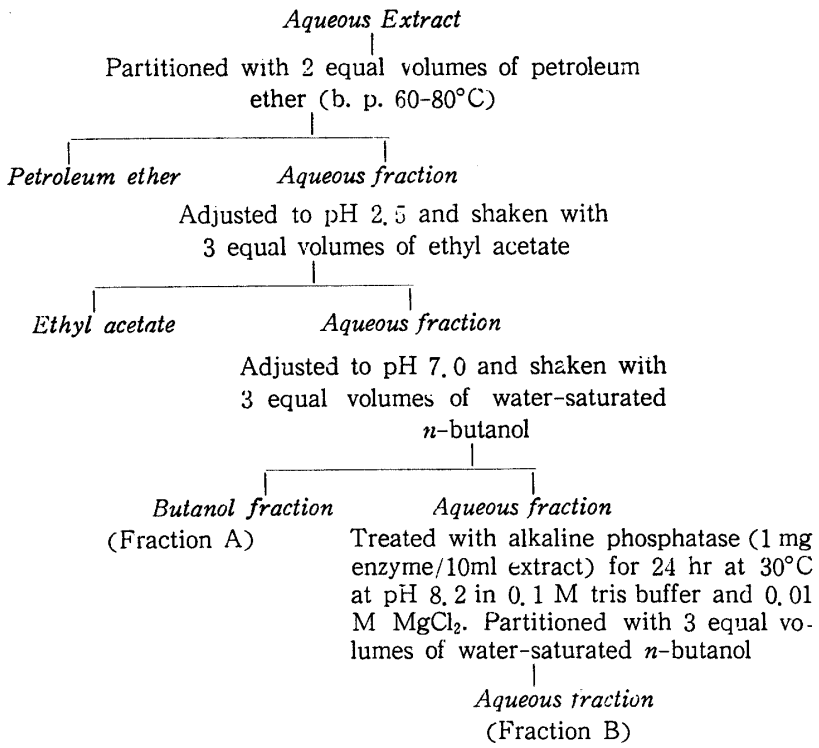

Fig. 1. Extraction procedure for cytokinin from gladiolus corms.

which were then immersed in 200 ml of 90% methanol at 20°C for 48 hours. The immersing solvent was evaporated *in vacuo* at 50°–55°C to obtain 50 ml of aqueous solution.

Eluated from the chromatogram were added to the basal medium at a concentration equivalent to 400 g of fresh corm per liter. The Miller's method (1962) was adapted in bioassay of the cytokinin-like activity.

2 ; *Determining the internal condition for sprouting in gladiolus corm.*

Corms of gladiolus cultivar 'Hector' were harvested on October 5, 1973. They were allowed to dry at room temperature for 2 weeks, and then were stored under one of the following conditions; (1) at 20°C constant for 5 weeks, (2) at 0°C for 1, 2, 3, 4 or 5 weeks, (3) at 30°C for 1 or 2 weeks after low temperature storage for 5 or 6 weeks. Tissue blocks, 0.2× 0.2×0.2 cm in size, each carrying terminal buds, were aseptically isolated from the corms and planted on Murashige and Skoog medium (1962) with or without 1 ppm benzyladenine. The culture temperature was 25°C and the lighting condition 5,000 lux.

Interrelationship between sprouting of buds and the size of tissue of corm carrying them, was examined. Plant materials similar to those in the previous experiment were used. Three kinds of blocks, 0.2 cm cube, 0.5 cm cube and 1 cm cube in size, were prepared, which hereafter will be referred to as the S-bud, M-bud and L-bud, respectively. The blocks each carrying the terminal bud were excised from the corms stored and were cultured under the same condition as in the previous experiment except for the culture condition. The blocks excised from corms immediately after harvest were cultured at 5°C for 5 weeks and then at 25°C for 2 months.

3 ; *Molecular action of cytokinin and inhibitors in relation to the dormancy of gladiolus corm.*

Corms of gladiolus cultivar 'Hector', 20-30 g in weight were used in this experiment.

Plugs (0.5×0.5×0.5 cm) of the corm tissue each carrying an apical bud were excised, and 10 plugs (0.8 g) were placed on filter paper in a Petri dish (9 cm in diameter). Then, a solution containing 0.6 ml of 3.9×10^{-4}M benzyladenine (BA)-8-^{14}C (12.8 mCi/mM) and 11.4 ml of water was added. In another treatment a solution containing 0.6 ml of ^{14}C-BA (3.9×10^{-4}M), 6 ml of abscisic acid (ABA) (10^{-4}M), 1.2 ml pyrogallol (10^{-3}M), 1.2 ml of coumarin (10^{-3}M) and 3 ml of water was added into Petri dish. Each treatment was duplicated.

After the buds were incubated with ^{14}C-BA at 20°C for 24 hours, they were transferred to the radioactive-free medium containing only water. Then, they were incubated at 20°C for 0, 24, 48 or 72 hours in darkness.

Yasuda's method (1971) was followed in fractionation. The buds thus incubated were homogenized with 6 ml of buffer solution (0.05 M tris-HCl, 0.5 M sucrose, 0.01 M $MgCl_2$, 0.001 M Na_2EDTA, 0.02 M KCl and 0.005 M mercapt ethanol) under a cold condition. The homogenate was centrifuged at 2,000 g for 20 minutes. After pouring out the supernatant, the residue was stirred with 4 ml of the buffer solution and then centrifuged. The residue thus obtained was designated as Fraction 1. The supernatants were combined and centrifuged at 20,000 g for 30 minutes. Thus, another residue (Fraction 2) and a supernatant (Supernatant 1) were obtained. Supernatant 1 was filled up to 15 ml with the buffer solution (Fraction 3). Fraction 3 (0.2 ml) was spotted on glass fiber paper (Whatman GF/C). Eight milliliter of 5% TCA (trichloroacetic acid) was added to 2 ml of Fraction 3. This mixture was cooled for 20 minutes, and was centrifuged at 2,000 g for 15 minutes. The supernatant was filtered on GF/C, and the residure obtained on it was designated as Fraction 4.

Portions of Fraction 3 were also treated with DNase or RNase (manufactured by Nagase and Co. Ltd.). All the enzymes were used at a concentration of 0.5 mg/ml. One milliliter of Fraction 3 was treated with each enzyme in 1 ml of 0.01 M sodium phosphate buffer (pH 6.0) for 60 minutes. After the enzyme treatment, 10% TCA was added into each solution. These solutions were filtered on GF/C, and radioactivity of each residue was measured with the Beckman LS 100 liquid scintillation spectrophotometer. Each figure of radioactivity thus obtained was calculated to compare with that of Fraction 4. The difference in radioactivity between the DNase-treated fraction and Fraction 4 was used for the radioactivity of DNA fraction. The radioactivity of RNA and protein fraction was similarly expressed.

Results

1 ; The cytokinin activity was found in Fraction B of the extract from gladiolus corms, while Fraction A showed little sign of the activity. The highest cytokinin activity, which was indicated by the heaviest callus block was found in Fraction B of the extract from the corms stored at 0°C for 5 weeks (low temperature storage) followed by storing at 30°C for 10 days. The cytokinin activity was present at Rf. 0.6-0.8. The activity at Rf. 0.7 was equivalent to approximately 0.1 mg/l kinetin (Figs. 2 and 3). In Fraction B of the extract from the corms stored at 0°C for 5 weeks and then at 30°C for 10 days, the cytokinin activity was a little higher than in those stored at 20°C for 5 weeks, but was less than that of Fraction B (Fig.4).

These results indicate that the endogenous cytokinin activity in the corm increased

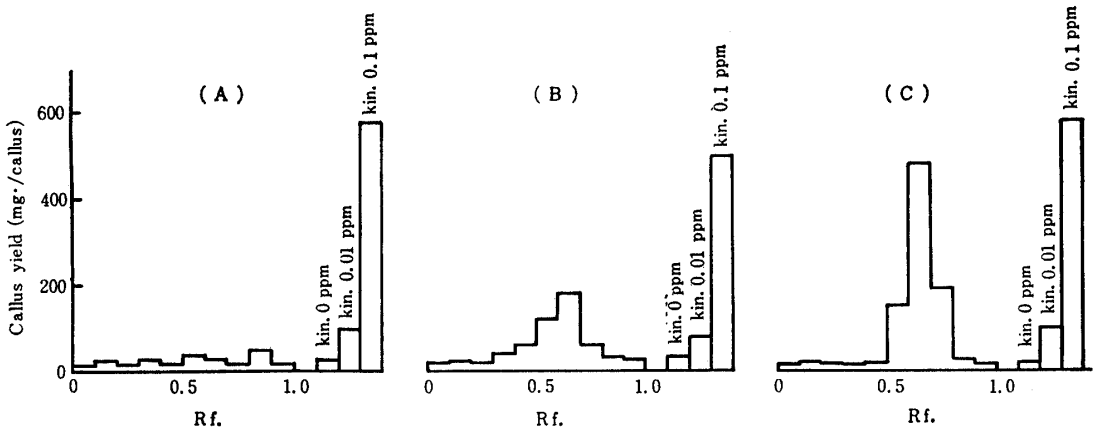

Fig. 2. Change in the level of cytokinin in Fraction B of gladiolus corms (cv. Hector).
A: 20°C for 5 weeks
B: 0°C for 5 weeks
C: 0°C for 5 weeks followed by 30°C for 10 days

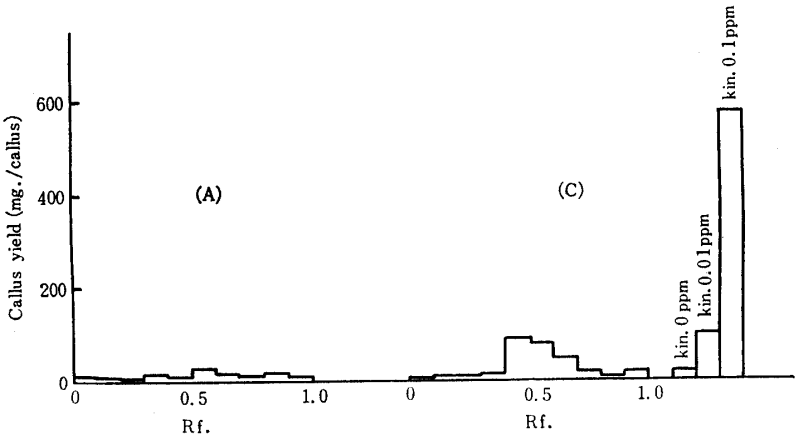

Fig. 3 Change in the level ot cytokinin in Fraction A of gladiolus corms (cv. Hector).
A: 20°C for 5 weeks
C: 0°C for 5 weeks followed by 30°C for 10 days

rapidly when the corm was transferred to high temperature condition following low temperature.

2 ; As shown in Table 1 and Figs. 5 and 6, most of the buds on blocks (0.2×0.2×0.2 cm in size) isolated from the corms which had been stored at 0°C for 1, 2, 3, 4 or 5 weeks, and at 20°C for 5 weeks, failed to sprout. In contrast, 90 to 100% of the buds on blocks isolated from the corms treated with high temperature for 2 weeks following the low temperature storage, sprouted. And those isolated from corms treated with high temperature for 1 week after low temperature storage did not sprout.

In all cases, however, the buds sprouted within 2 weeks of culture when 1 ppm BA was added to the basal medium.

S-, M- and L-buds isolated from corms immediately after harvest, did not sprout even after 4 months of culture, as shown in Table 1 and Figs. 7-1 and 7-2. Although S- and M-buds isolated from corms immediately after low temperature storage did not sprout, only L-

bud sprouted 3 weeks after explanting. Even when S-, M- and L-buds isolated immediately after harvest, were stored at 25°C for 2 months following storage at 5°C for 5 weeks in test tubes, they did not sprout.

3 ; Percentage of radioactivity incorporated into Fraction 1 is shown in Fig. 8. Cell wall fragments, starch grain, etc. were included in this fraction. There was no difference in percentage of radioactivity in the fraction between the extract from buds treated with ^{14}C-BA alone, and that with the combined solution of ^{14}C-BA and inhibitors.

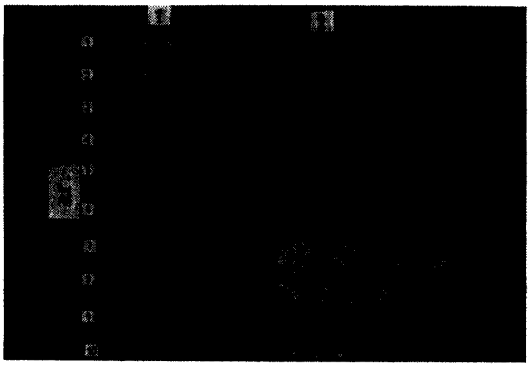

Fig 4. Comparison of growth of soybean callus on cultural medium containing respective eluate from paper chromatogram segments of Fraction B (after culturing for 21 days).
1 : 20°C for 5 weeks
2 : 0°C for 5 weeks followed by 30°C for 10 days.

Table 1. Comparison of sprouting of S-buds, isolated from corms stored under various conditions, on the medium with or without BA (4 weeks after explanting).

Treatment	Percent sprouting
*Low temperature 1 week	0
〃 2 weeks	0
〃 3 weeks	30
〃 4 weeks	0
〃 5 weeks	10
Low temperature 5 weeks +30°C 1 week	20
Low temperature 6 weeks +30°C 1 week	10
Low temperature 5 weeks +30°C 2 weeks	90
Low temperature 6 weeks +30°C 2 weeks	100
**Low temperature 5 weeks +BA 1 ppm	100
Low temperature 6 weeks +BA 1 ppm	100

*M- and L-buds did not sprout
**M- and L-buds all sprouted

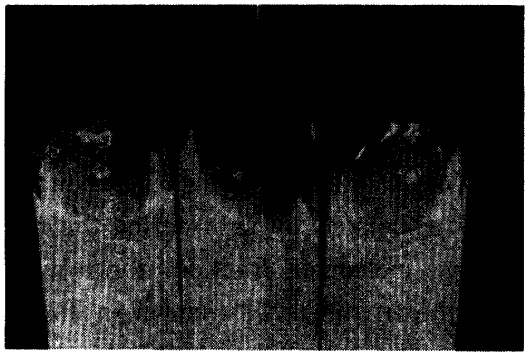

Fig. 5. S-buds isolated from gladiolus corms stored at 0°C for 4, 5 and 6 weeks (from left to right, 2 months after low temperature storage of corms).

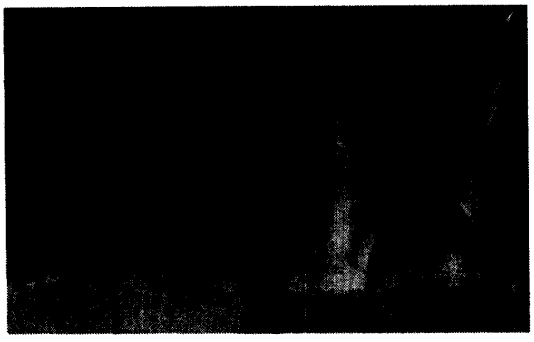

Fig. 6. Sprouting of S-buds isolated from gladiolus corms stored at 0°C for 5 weeks on the medium with (the two on the right) and without (the two on the left) 1 ppm benzyladenine (4 weeks after explanting).

Fig. 7—1. Sprouting of L-, M- and S-buds (from left to right) isolated from gladiolus corms stored at 0°C for 5 weeks (4 weeks after explanting).

Fig. 7—2. L-, M- and S-buds isolated from gladiolus corms immediately after harvest, then stored *in vitro* at 5°C for 5 weeks and cultured at 25°C for 45 days.

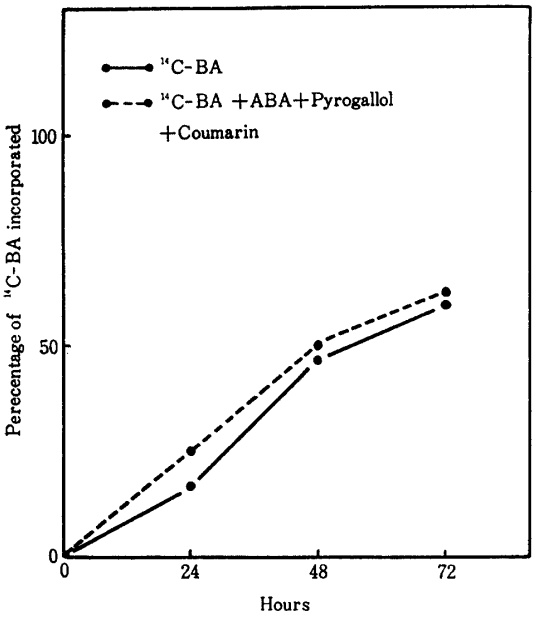

Fig. 8. Effect of inhibitors on percentage of radioactivity incorporated into Fraction 1.

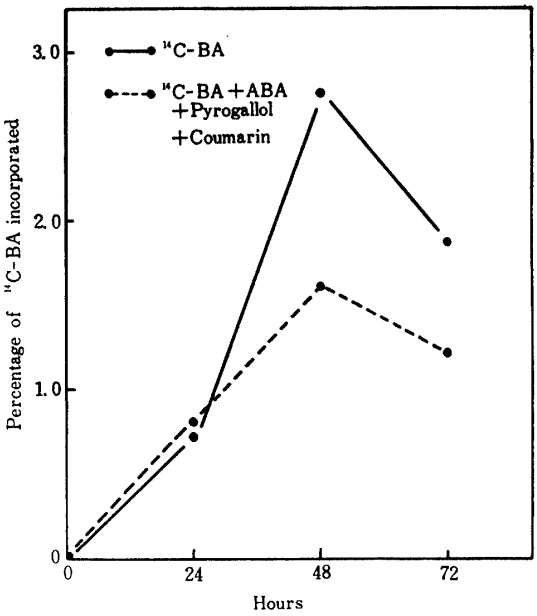

Fig. 9. Effect of inhibitors on percentage of radioactivity incorporated into Fraction 2.

As shown in Fig. 9, 24 hours after incubation the radioactivity in Fraction 2 of the extract from the bud treated with ^{14}C-BA was slightly higher than that from the bud treated with the combined solution of ^{14}C-BA and inhibitors. This fraction contains mitochondria.

The radioactivity incorporated into low molecules was calculated by subtracting the radioactivity in Fraction 3 from that in Fraction 4. In this low molecular fraction, there was no difference in radioactivity between the extracts of both the treatments as shown in Fig. 10.

Percentage of radioactivity in Fraction 4, 24 hours after feeding of ^{14}C-BA, which contained high molecular substances such as DNA, RNA and protein, did not differ between the extract from the bud treated with ^{14}C-BA and that treated with ^{14}C-BA and inhibitors. Forty-eight or 72 hours after feeding of ^{14}C-BA, however, percentage of radioactivity in this fraction increased by the treatment with ^{14}C-BA at a higher rate than by the combined

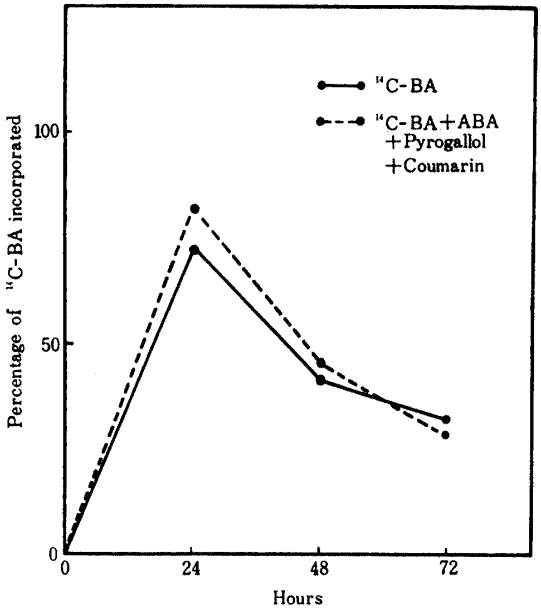

Fig. 10. Effect of inhibitors on percentage of radioactivity incorporated into the low molecular fraction.

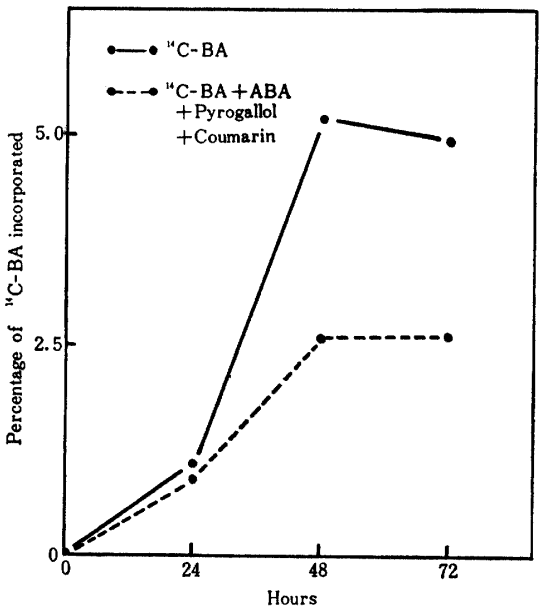

Fig. 11. Effect of inhibitors on percentage of radioactivity incorporated into the high molecular fraction.

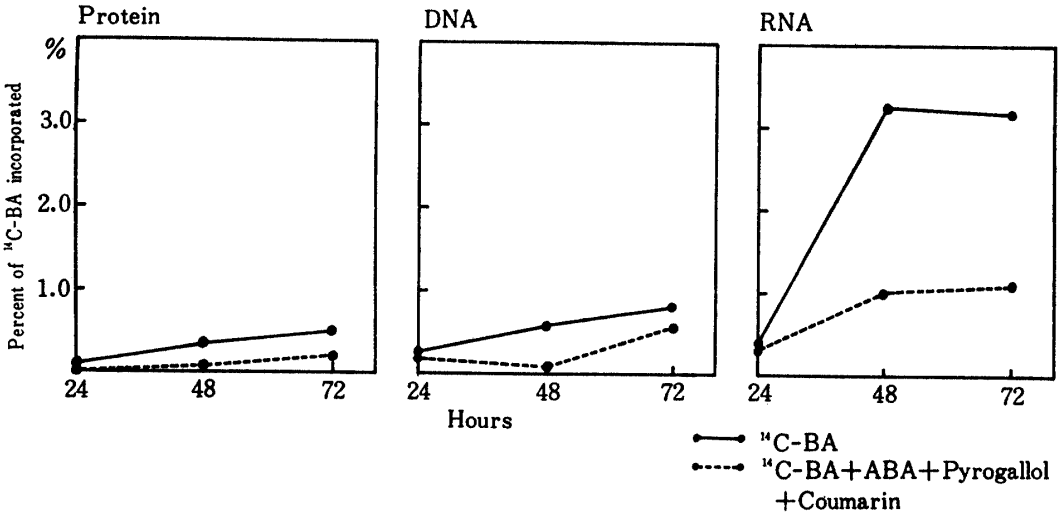

Fig. 12. Effect of inhibitors on percentage of radioactivity incorporated into protein, DNA and RNA fraction.

treatment with ^{14}C-BA and inhibitors (Fig. 11).

Percentage of radioactivity incorporated into DNA, RNA and protein was compared in Fig. 12. Among DNA and protein of the extracts from the bud treated with ^{14}C-BA and with the combined solution of ^{14}C-BA and inhibitors, there was no difference. However, percentage of radioactivity in RNA fraction of the extract from the bud treated with ^{14}C-BA was higher than that treated with ^{14}C-BA and inhibitors, 48 and 72 hours after incubation.

Discussion

In our previous studies (1972c, 1975), cytokinin was effective in breaking dormancy of gladiolus and freesia corms and potato tubers. Notwithstanding that many workers have studied the role of cytokinin in breaking dormancy of some plants, there remain many problems unsolved at present.

The idea that growth-inhibiting substances might have a regulatory function in dormancy, was first suggested by Molish (1922). Since then, a considerable amount of evidence has been accumulated, indicating that a larger amount of inhibitor occurs in a dormant than that in a nondormant plant.

On the gladiolus corm, whose dormancy is broken with low temperature, Tsukamoto (1959, 1972b) reported that the level of endogenous growth inhibitors was reduced when corms were exposed to low temperature. It was recognized, however, in our previous paper (1975) that endogenous acidic growth inhibitors did not decrease even when the dormancy was broken with cytokinin. So we examined the antagonistic relationship between cytokinin and exogenous inhibitors (abscisic acid etc.), and could clarify the antagonistic relationship (1975). Then, Khan et al. (1964, 1965, 1971) reported that the germination in lettuce seeds was completely inhibited by the naturally occurring inhibitors, coumarin, xanthine, abscisic acid and a partially purified inhibitor from immature wheat hulls, and inhibition of germination with these chemicals could be reversed by cytokinin.

Based on these results, it might be concluded that the release from dormancy when treated with exogenous cytokinin, is not caused by decrease in the amount of inhibitors but by the obstruction of inhibitor activities due to cytokinin application. And therefore, changes in the cytokinin activity in the corm through the dormancy releasing process caused by low temperature treatment were examined in the present study. The activity of the endogenous cytokinin was hardly detected in the corms immediately after the low temperature storage for 5 weeks, although this treatment was sufficiently effective of breaking the dormancy. However, when the corm was further stored at a high temperature for 10 days, cytokinin activity in the corm rapidly increased. From these results, it seems possible to postulate that the procedure of cytokinin synthesis in the corm is prepared by exposure to a low temperature and actual cytokinin synthesis is triggered by a high temperature following exposure to a low temperature.

Explants of small and medium sizes excised from corms having been stored at low temperature for several weeks did not sprout, while those excised from corms having been stored further at high temperature for 2 weeks, sprouted after *in vitro* culture for 2 weeks on the medium. On the other hand, when cytokinin was supplemented to the medium these explants of small and medium sizes could sprout, even if they were taken from corms immediately after harvest or low temperature storage. This supports the result of changes in endogenous cytokinin level in the prior experiment. Meanwhile in the experiment of *in vitro* culture of buds, larger sized explants could sprout on the medium without BA within 2 weeks after culture at 25°C even when they were taken from corms immediately after low temperature storage. This fact suggests that, for the corm tissue attached to the bud to respond to high

temperature and therefore to synthesize cytokinin, its size must be above a critical one. These data, however, fail to clarify which relates the cytokinin synthesis, the size of cambium included in the excised tissue or that of root initials.

There are a few papers reporting that the release of the gladiolus corm from dormancy following exposure to low temperature is caused through a decreased amount of endogenous growth inhibitors. This decrease of inhibitors alone, however, does not appear to be a sufficient condition for release of the corm from dormancy, because in the present experiment of *in vitro* bud culture, the smaller explant taken from corms immediately after low temperature exposure could not sprout unless cytokinin was supplemented into the culture medium.

It is thus concluded that cytokinin synthesis induced by high temperature following an exposure to low temperature is requisite for establishing the necessary state for sprouting of gladiolus corms.

Furthermore, aiming at clarifying this antagonistic relationship between cytokinin and inhibitor in breaking dormancy of the gladiolus corm, molecular action of cytokinin and inhibitors was investigated in the present study using ^{14}C-labelled benzyladenine. As the result, it was shown that incorporation of ^{14}C-BA into cell wall, mitochondria, low molecule, protein and DNA of the corm was little if any influenced by the inhibitors, and that its incorporation into RNA was inhibited when the corm was incubated with ^{14}C-BA, abscisic acid, pyrogallol and coumarin. It seems that the antagonism between cytokinin and inhibitors on breaking dormancy is related to RNA metabolism. In Fox's (1967) and Matsubara's (1968) reports on microorganism (*E. coli* and corynebacterium), it was recognized that cytokinin labelled with ^{14}C was incorporated into t-RNA. But Miernyk (1975) reported that in germinating lettuce seed ^{14}C-kinetin was incorporated into riboside and AMP, though there was no detectable incorporation of kinetin into t-RNA. Thus there remains much difficulty to be solved in studying the metabolism of cytokinin on germination and sprouting of the plant.

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要旨 サイトカイニン類が種々の球根植物の休眼打破に効果があることは，塚本らがすでに報告している。本報告では，グラジオラス球茎の休眠覚醒にともなう内生サイトカイニンの消長とその作用機構を検討した。

一般に認められているグラジオラス球茎の休眠打破に必要な低温処理を行っても，処理直後では内生サイトカイニンの活性化はほとんど認められなかったが，低温処理後10日間高温（30°C）下においた球茎では内生サイトカイニン活性の著しい増加が認められた。

種々の条件で貯蔵した球茎の頂芽部（0.2 cm^3）を無菌培養し，その発芽状態を調べたところ，低温処理直後の球茎の頂芽部は発芽しないが，低温処理後 30°C に2週間貯蔵した球茎の頂芽部は培養3週間でほぼ100％の発芽率が認められた。一方低温処理直後の球茎の頂芽部を 1 ppm ベンジルアデニンを含む培地で培養するとすべての個体が発芽した．頂芽部の培養組織片の大きさを大（1cm^3），中（0.5cm^3），小（0.2cm^3）に分けて培養したところ，大きい組織片では低温処理直後に培養しても発芽するが，中および小さい組織片では低温処理後10～15日間 30°C に貯蔵した球茎でないと発芽しなかった。また，収穫直後の球茎から大，中，小の頂芽部組織片を切りとり，試験管内で5週間低温処理を行い，以後 25°C で45日間培養しても発芽する個体は認められなかった。

つぎに，ベンジルアデニン-8-^{14}C を用い，サイトカイニンとグラジオラス球茎の発芽抑制物質（アブシジン酸，ピロガロール，クマリン）の組合せ処理による，サイトカイニンの球茎の休眠打破における作用機作を検討した。その結果，抑制物質と ^{14}C-ベンジルアデニン混合処理区での ^{14}C の RNA へのとりこみ率が ^{14}C-ベンジルアデニン単独処理区にくらべて非常に低かった。このことから抑制物質とサイトカイニンがＲＮＡ代謝に関与して，グラジオラス球茎の休眠を制御しているものと思われる。