イネカルスの緑化部位形成に対するオーキシンとサイトカイニンの影響

誌名	日本作物學會紀事
ISSN	00111848
著者	井上, 貢 前田, 英三
巻/号	49巻2号
掲載ページ	p. 167-174
発行年月	1980年6月

Japan. Jour. Crop Sci. 49(2) : 167-174 (1980)

Effects of Auxins and Cytokinins on the Occurrence of Green Regions in Rice Callus Cultures

Mitsugu INOUE and Eizo MAEDA

(Institute for Biochemical Regulation, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya, 464 Japan),

Received September 5, 1979

Since the kinetin/auxin ratio in the medium was indicated as a factor effective on organ formation in tobacco callus cultures[18], attempts to determine the organogenetic effect of these substances are widespread with many plant species. In rice callus, shoots, roots and plantlets were formed in the medium containing lower concentrations of auxin[3,5,10,14]. An addition of kinetin promoted the shoot formation[16]. But whether the cytokinin/auxin ratio is essential for organogenesis in rice callus and whether a limited amount of auxin is required for it have been remained as to be solved.

To answer these questions, we aimed to clarify the effects of auxins, cytokinins and an antiauxin on the greening and organogenesis in rice callus. Under light condition, entire surface of tobacco callus was usually covered with chlorophyllous green color[11], while in rice and barnyardgrass several regions with green color were scattered on the callus surface in the organ forming medium. To reveal those events occurred in organogenesis of rice callus, the green regions were especially selected. Because (1) intimate relationships between the green regions and shoot formation have been indicated[12,13] and (2) the green regions occur extremely more than shoots on a callus, facilitating a quantitative comparison.

To find the appropriate materials, preliminary tests were made on the effect of the number of subculture and the texture of callus on the greening and organogenesis. The greening in barnyardgrass callus was also examined for comparison with rice callus.

The results obtained in this research will be suggestive for the mechanism of greening and organ formation in rice callus and furthermore of greening and senescence of leaves in rice plants.

Materials and Methods

The callus was induced to form directly from rice (*Oryza sativa* cv. Aichi Asahi) and barnyardgrass (*Echinochloa crus-galli*) seeds as described previously[3]. Maeda's medium containing 10^{-5}M 2,4-D (2,4-dichlorophenoxyacetic acid) and 9 g/l agar[3] was used as a subculture medium except described especially. The medium containing 10^{-7}M 2,4-D and 5×10^{-5}M kinetin was used as an organ-forming medium. According to the purpose of the experiments, 2,4-D, NAA (1-naphthaleneacetic acid), TIBA (2,3,5-triiodobenzoic acid), kinetin, zeatin and isopentenyladenosine were added to Maeda's medium with various concentrations. The callus was subcultured every about 30 days at 30°C in continuous light (about 200 lx), transferred to various media and cultured at 25°C in continuous light (about 3,000 lx). Two pieces of callus (each about 100 mg fresh weight) serve as inoculum in Erlenmeyer flasks containing 50 ml of the medium.

The number of green regions, shoots and roots on the callus tissues were counted. Green spots usually appeared about 1 to 5 in each green region. Therefore, the number of the spots on each region was counted

A part of this work was reported at the 3 rd symposium for Plant tissue culture held in Nagoya, July, 1972 and at the 158 th meeting of the Crop Sci. Soc. of Japan held in Hirosaki, October, 1974.

and the total number was recorded for precise observation. Sometimes, the number of flasks containing the callus with green regions or shoots were estimated. Chlorophyll determination was made according to Arnon's method[1] on the light absorption by aqueous acetone (80%) extracts.

Results

1. *Effect of subculture and callus texture*

Number of green regions and shoots decreased as the number of subculture increased (Table 1). Neither shoot nor root were observed in the 7th passage. Abnormal plants often regenerated from the callus after the 4th passage. Albino plants and plants with abnormal hulls with a few of trichomes and/or the absence of an awn were found (Fig. 1). In the subsequent experiments, therefore, the rice callus in the 2nd passage was used to transfer.

Effects of friability in the inoculated callus on the differentiation in rice callus are shown in Fig. 2. The callus in the 2nd passage was separated into compact or friable callus according to the texture and the respective callus was transferred to the organ-forming medium. Green regions and shoots were observed in the compact callus more than in the friable callus. Thus, the compact callus was selected for the subsequent experiments.

Table 1. Effect of progressive subculture on organ formation in rice callus. Number of green regions, shoots and roots per 20 flasks was determined 24 days after the callus was cultured on the organ-forming medium.

Passage*	Green region	Shoot	Root
1**	1180	68	4
2	580	5	19
3	250	1	8
4	150	1	2
5	100	0	5
6	40	0	1
7	27	0	0

*Number of passage before transfer to the organ-forming medium.

**The callus transferred to the organ-forming medium immediately after induced from a seed.

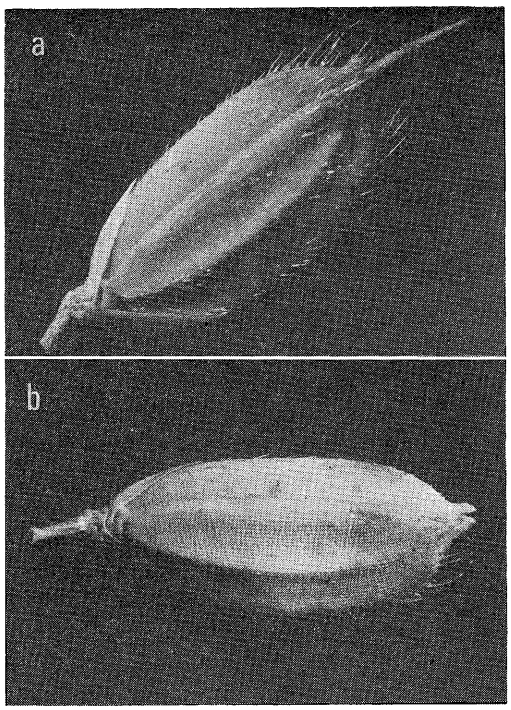

Fig. 1. Anomalous morphology of a seed (b) produced on a plant that regenerated from the callus culture, as compared with a seed (a) of a rice cultivar that was used for callus induction (×6.7).

From the comparison with absorption spectra in acetone extracts of the callus tissues with or without green regions and of the rice leaves, it was found that the chlorophyll composition in the callus with green regions was similar to that in leaves while any peak owing to chlorophyll was not present in the callus without green regions (Fig. 3). Therefore, it can be said that the formation of green regions is an aspect of the development of chloroplasts in rice callus.

2. *Effect of cytokinin and auxin*

All cytokinins used; kinetin, zeatin and isopentenyladenosine, increased the number of green regions in rice callus (Table 2). Zeatin being a major cytokinin in rice callus[8] was especially active.

The effect of the combination of 2,4-D and kinetin on the number of green regions

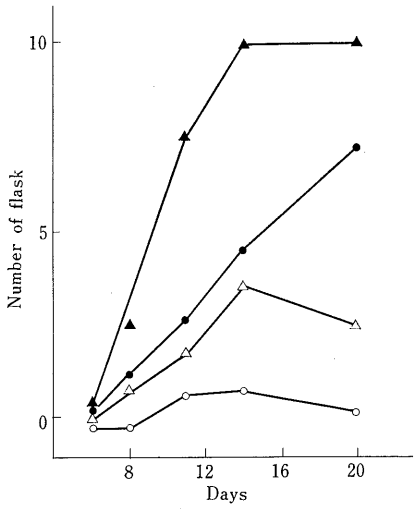

Fig. 2. Effect of callus texture, compact (triangle) or friable (round), on the number of flasks contained the rice callus forming green region (black) or shoot (white). Ten flasks were used for each treatment.

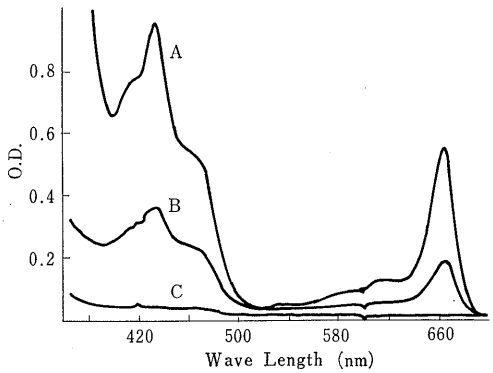

Fig. 3. Comparative absorption spectra in acetone extracts of rice leaves (A) in seedlings that were grown for 15 days at 30°C under light condition (about 3,000 lx) and of rice callus tissues with (B) or without (C) green region that were cultured for 20 days on the organ-forming medium.

is shown in Fig. 4, indicating the increment of the number at lower concentration of 2,4-D and higher concentration of kinetin. The pronounced effect by higher concentration of kinetin was obtained at 10^{-7}M, 10^{-8}M, 10^{-9}M and the absence of 2,4-D. At 10^{-6}M of 2,4-D, higher concentration of kinetin was slightly effective. No effect of kinetin was induced at 10^{-5}M of 2,4-D. In the kinetin-omitted media, a promotive effect was equally obtained at 10^{-8}M, 10^{-9}M and the absence of 2,4-D. In the relation to external morphology, lower concentration of 2,4-D and higher concentration of kinetin allowed the callus texture to change into compact.

In this results, however, no evidence was obtained to show the necessity of the presence of auxin at lower levels for the increment in the green regions. To clarify whether auxin is really required for it or not, an influence of auxin in the preculture medium and an action of TIBA were ascertained.

Since 2,4-D is more active auxin in relation to the callus induction than IAA and NAA[3] and scarcely decomposed in the callus cells[6], the carry-over effect of 2,4-D from the preculture medium by the inoculated callus should be considered. Thus, to evaluate the effect of the auxin in the inoculated callus on the appearance and the number of the green spots, 2,4-D in the preculture medium was replaced by a weak auxin *i.e.* NAA and the callus was transferred to the auxin-free medium with various levels of kinetin. The result represented that the

Table 2. Effect of cytokinin on number of green regions in rice callus. The number (per 20 flasks) was determined 20 days after the callus was cultured on the medium containing one of cytokinins indicated below in the presence of 2, 4-D at 10^{-7}M. The number was 154 in cytokinin-free medium.

Cytokinin	Concentration (M)		
	10^{-5}	10^{-6}	10^{-7}
Kinetin	482	250	194
Zeatin	655	381	307
Isopentenyladenosine	465	265	145

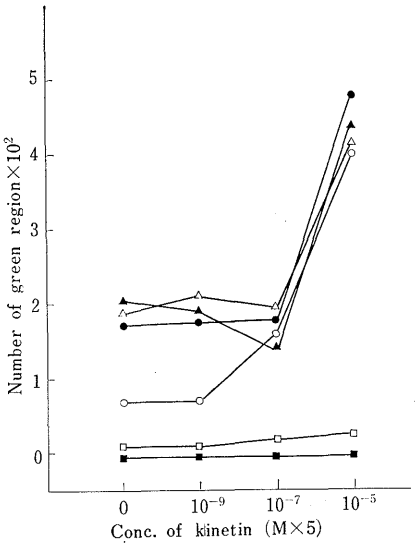

Fig. 4. Effect of concentration of 2,4-D and/or kinetin on the number of green regions appeared on rice callus. The number of green regions per 20 flasks was determined 22 days after transfer to the media containing various concentrations of 2, 4-D and/or kinetin. Concentration of 2,4-D (M) ; ■ ; 10^{-5}, □ ; 10^{-6}, ○ ; 10^{-7}, △ ; 10^{-8}, ▲ ; 10^{-9}, ● ; none.

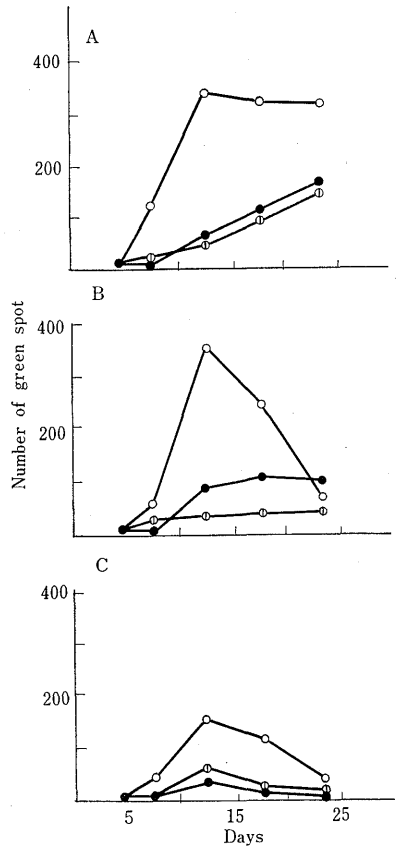

Fig. 5. Effect of kinetin concentration on the number of green spots appeared on rice callus. The callus was transferred to the auxin-free media containing various concentrations of kinetin after precultured for 20 days on the media containing various concentrations of NAA. Concentration of NAA (M) : A ; 10^{-4}, B; 10^{-5}, C ; 10^{-6}. Concentration of kinetin (M) : ○ ; 5×10^{-5}, ⦶ ; 5×10^{-7}, ● ; none. Ten flasks were used for each treatment.

higher the concentration of NAA in the preculture medium was, the more the number of spots was (Fig. 5). Kinetin was apparently effective at 5×10^{-5}M on the increment but not at 5×10^{-7}M. When the callus were transferred from the preculture media containing 10^{-6}M and 10^{-5}M NAA to the 5×10^{-5}M kinetin media, as the culture time proceeded, the number of green spots represented a rise followed by a fall, changing the color of spots to brown and it is of the largest at 12 days after transfer. In addition, the length of lag period for appearing the spots and the number of these spots increased by the preculture at 10^{-5}M 2,4-D than 5×10^{-4}M NAA (Fig. 6). Hence, the apparent involvement of auxin, especially carry-over effect of auxin from the preculture, can be suggested in the greening on rice callus.

To further test the auxin involvement, an effect of TIBA, an antiauxin, was examined on the occurrence of green regions. It was inhibited by the higher concentration of TIBA (Fig. 7). However, the fresh weight of callus was not repressed by 10 ppm of TIBA, at which concentration the occurrence was affected (Table 3). Therefore, it is likely that the repression by TIBA of the occurrence in the green regions was not caused by the decrease in callus growth. Thus, in the experiment using an antiauxin, the participation of auxin for the greening was also confirmed in rice callus.

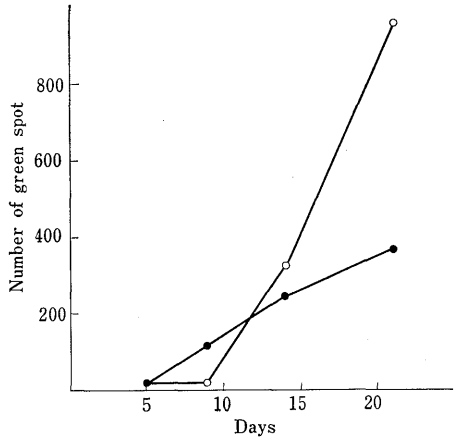

Fig. 6. Effect of 5×10^{-5}M kinetin on the number of green spots appeared on rice callus. The callus was transferred to the auxin-free medium containing 5×10^{-5}M kinetin after precultured for 20 days on the media containing 10^{-5}M 2,4-D (○) or 5×10^{-4}M NAA (●). Ten flasks were used for each treatment.

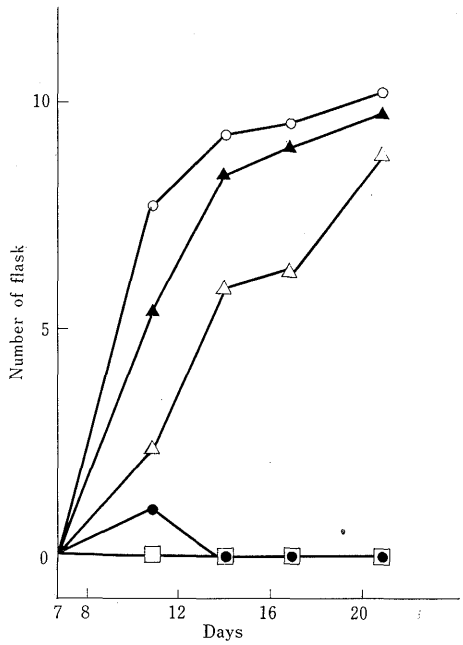

Fig. 7. Effect of TIBA concentration on the number of flasks contained the rice callus forming green region. The callus was transferred to the organ-forming media containing various concentrations of TIBA. Concentration of TIBA (ppm): □; 100, ●; 50, △; 10, ▲; 1, ○; none. Ten flasks were used for each treatment.

For detailed research on the effects of auxin and cytokinin on the greening, lag period for inducing green regions was examined in relation to the concentration of 2,4-D and kinetin in the culture medium. Table 4 shows the lag period for the appearance of the green regions together with that of adventitious roots. When the callus were transferred to the media containing 10^{-5}M of 2,4-D and various levels of kinetin, neither the green region nor the adventitious root were detected during 30 days of culture period. But both were early formed at lower concentrations of 2,4-D and higher concentrations of kinetin. Thus, it is suggestive that the induction of the greening and rhizogenesis in rice callus chiefly depends upon the levels of auxin and cytokinin in the culture medium. On the other hand, it has shown that the length of lag period for callus induction from the rice seeds and the segments excised from rice seedlings shortened, when auxin concentration in the medium was increased[3,4].

To compare with rice callus, barnyardgrass callus was also tested (Table 5). The green region on barnyardgrass callus was found 3 days after the transfer to the auxin-omitted medium and even 7 days after the transfer to the medium containing 10^{-5}M 2,4-D, at which concentration no occurrence of the green regions on rice callus was detected. In this case, no apparent effect

Table 3. Effect of TIBA added to the organ-forming medium on the growth of rice callus cultured for 35 days.

Concentration of TIBA (ppm)	Fresh weight* (g)
0	1.82±0.07
1	1.92±0.08
10	1.72±0.07
50	1.32±0.04
100	1.15±0.04

*Results are expressed as mean values with standard deviations (10 flasks).

Table 4. Lag period for the formation of green region (G) or adventitious root (R) in rice callus as affected by different concentration of 2,4-D and kinetin. Figures in the table indicate the first appearance of green region or adventitious root as indicated by number of days after inoculation.

2,4-D (M)		Kinetin (M ×5)			
		10^{-5}	10^{-7}	10^{-9}	0
10^{-5}	G	—	—	—	—
	R	—	—	—	—
10^{-6}	G	19	23	23	23
	R	19	19	19	19
10^{-7}	G	7	7	11	11
	R	7	7	7	11
10^{-8}	G	7	9	9	12
	R	7	9	7	11
10^{-9}	G	7	7	12	12
	R	7	7	10	11
0	G	7	7	7	11
	R	5	8	8	7

— Sign indicates callus without green region or adventitious root. Twenty flasks were used for each treatment.

was obtained by kinetin.

Discussion

It has been reported in tobacco callus[18] that low, intermediate and high kinetin-to-IAA ratios favored root formation, undifferentiated callus growth and bud formation respectively. The relative ratios were also required for differentiation and organogenesis in *Narcissus*[17], and cytokinins were remarkably effective on the occurrence of green regions which led shoots in rice callus[12,16]. However, the kinetin-to-auxin ratio alone is not probable to relate with the occurrence of the green region in rice callus, because of more production of the regions with the five-to-one ratio (5×10^{-7}M kinetin to 10^{-7}M 2,4-D) than the fifty-to-one ratio (5×10^{-5}M kinetin to 10^{-6}M 2,4-D) as shown in Fig. 4.

The length of lag period for the first appearance of green region and adventitious root was found to decrease in lower levels of auxin in the culture medium. The decrease in the length was also shown when 2,4-D in preculture medium was replaced by a weak auxin. These results suggest that the decrease in auxin level favored the differentiation in rice callus. This opinion has been supported by several findings[3,10,14]. However, the carry-overed auxin accelerated the greening in rice callus. An antiauxin, TIBA, repressed the occurrence of green regions. From these results, it can be concluded that not only the cytokinin-to-auxin ratio but also a limited amount of auxin may be important in the greening of rice callus. Beside rice callus, a slight amount of auxin may be also required for differentiation in other plant species which the differentiation was induced as auxin concentration alone was decreased in the culture medium[2,19].

In addition, the greening was more evident in the barnyardgrass callus than the rice callus. It is true that the appearance of this histological differentiation in plant callus intimately associates with the decrease in the auxin level caused by the metabolism in the callus cells, because the decomposition of 2,4-D was more remarkable in the barnyardgress callus than the rice callus[6]. Hence a special attention must be paid for the amounts of phytohormones in the callus tissues as same as in the medium, because of the metabolic change in callus tissues and of the diffusion of auxin to the medium.

Thiamine[7] and casein hydrolysate[9,16] were previously found to act not only individually but also in the combination with kinetin on the differentiation in rice callus. Consequently, it is probable that mutual relationships among the physiologically active substances are involved in the greening of rice callus.

As the subculture proceeded, the rice callus lost the ability for differentiation and the texture altered gradually from compact to friable. The friable callus was not favorable for the differetiation than the compact callus. As auxin concentration decreased and kinetin concentration increased, the callus became to be compact and the number of green regions increased. Therefore, some factors which make callus increase the friability seem to induce the

Table 5. Lag period for the formation of green region in barnyardgrass callus as affected by different concentration of 2,4-D and kinetin. Figures in the table indicate the first appearance of green region as indicated by number of days after inoculation.

Kinetin (M)	2,4-D (M)			
	2×10^{-5}	10^{-5}	10^{-7}	0
5×10^{-5}	—	7	2	2
0	—	7	3	3

— Sign indicates callus without green region. Twenty flasks were used for each treatment.

repression of greening on its surface.

Kinetin was effective on the greening in rice callus as well as chlorophyll retention in rice leaves[15,20]. However, auxin was essential for the greening in rice callus but not the chlorophyll retention in rice leaves[15,20]. This discrepancy may be brought by the difference in the auxin level, which are required for respective responses, and in the auxin supply to the tissues. From the point of these views, our results presented here will provide some useful clue for the study of greening in young leaves originated from shoot apices of rice plants.

Acknowledgements

We thank Mr. Tsuguo SUGIURA for his kind help during the experiments and also thank Mrs. Kazuko MAEDA for reading the manuscript.

Summary

The effect of phytohormones is indicated in the study of factors controlling the greening which is shown as a scattered appearance of the green regions on rice and barnyardgrass callus cultures. Evidence was obtained that implicated both the number of subculture and the friability of callus in the organ formation. Many abnormal plants developed from the subcultured callus cultures. Absorption spectra in acetone extracts of the callus with green regions represented the identical pattern to that of leaves of rice seedlings. Kinetin, zeatin and isopentenyladenosine increased the number of green regions. When 2,4-D was decreased in the concentration and kinetin was increased, the number of the green regions increased and the length of lag period for the first appearance of the regions decreased. The promotive effect of kinetin was modified by 2,4-D at various levels. When the concentration of NAA in the preculture medium increased, the number of the green spots was increased. The occurrence of the green regions was repressed as TIBA level was increased. It is suggested from these results that not only a cytokinin/auxin ratio in the medium but also the amount of auxin in callus tissues influence the differentiation of green regions in rice callus.

References

1. ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoxyloxidase in *Beta vulgaris*. Plant Physiol. **24**: 1—15.
2. HALPERIN, W. 1966. Alternative morphogenetic events in cell suspensions. Amer. J. Bot. **53**: 443—453.
3. INOUE, M. and E. MAEDA 1976. Effect of auxin concentrations on the callus induction from various organs of rice seedlings. Proc. Crop Sci. Soc. Japan **45**: 545—557.
4. ————•———— 1976. Relation between 2,4-D levels and callus initiation in the epidermal cells of scutellum and coleorhiza of rice. Proc. Crop Sci. Soc. Japan **45**: 637—638.
5. ————•———— 1977. Occurrence of adventitious roots from rice leaf sheaths treated with 2,4-D. Japan. Jour. Crop Sci **46**: 319—320.
6. ————•———— 1980. Absorption and metabolism of radioactive auxins in the induced rice callus. Japan. Jour. Crop Sci. **48**: 1—9.
7. ————•———— 1980. Thiamine as a factor of organ formation in rice callus cultures. Japan. Jour. Crop Sci. **49**: 1—7.
8. ————•———— R. YOSHIDA and T. ORITANI 1979. On the occurrence of a high content of cytokinins in rice callus tissues. Plant & Cell Physiol. **20**: 917—924.
9. KAWATA, S. and A. ISHIHARA 1968. The regeneration of rice plant, *Oryza sativa* L., in the callus derived from the seminal root.

Proc. Japan Acad. **44**: 549—553.
10. MAEDA, E. 1968. Subculture and organ formation in the callus derived from rice embryo in vitro. Proc. Crop Sci. Soc. Japan **37**: 51—58.
11. ———— and T. A. THORPE 1979. Shoot histogenesis in tobacco callus cultures. In Vitro **15**: 415—424.
12. NAKANO, H. and E. MAEDA 1974. Morphology of the process of shoot formation in the rice callus culture. Proc. Crop Sci. Soc. Japan **43**: 151—160.
13. ————•———— 1979. Shoot differentiation in callus of *Oryza sativa* L. Z. Pflanzenphysiol. **93**: 449—458.
14. NISHI, T., Y. YAMADA and E. TAKAHASHI 1968. Organ redifferentiation and plant restoration in rice callus. Nature **219**: 508—509.
15. ORITANI, T. and R. YOSHIDA 1969. Studies on nitrogen metabolism in crop plants. IV. Effects of several chemical substances and root extracts of rice plant on chlorophyll retention in rice leaf sections. Proc. Crop Sci. Soc. Japan **38**: 459—465.
16. SAKA, H. and E. MAEDA 1969. Effect of kinetin on organ formation in callus tissue derived from rice embryos. Proc. Crop Sci. Soc. Japan **38**: 668—674.
17. SEABROOK, J. E. A., B. G. CUMMING and L. A. DIONNE 1976. The in vitro induction of adventitious shoot and root apices on *Narcissus* (daffodil and narcissus) cultivar tissue. Can. J. Bot. **54**: 814—819.
18. SKOOG, F. and C. O. MILLER 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. **11**: 118—131.
19. STABA, E. J., P. LAURSEN and S. BUCHNER 1965. Medical plant tissue cultures. In "Proceedings of an international conference on plant tissue culture" edited by P.R. White and A. R. Grove P. 191—210. American Institute of Biological Science.
20. YAMADA, N., H. SUGE, H. NAKAMURA and K. TAZIMA 1964. Chemical control of plant growth and development. (5) Effect of kinetin and other chemicals on degradation of chlorophyll in rice plant. Proc. Crop Sci. Soc. Japan **32**: 254—258.

〔和 文 摘 要〕

イネカルスの緑化部位形成に対するオーキシンとサイトカイニンの影響

井 上 貢・前 田 英 三
（名古屋大学農学部生化学制御研究施設）

イネカルスの分化の中でもとりわけ緑化部位形成は著しく高頻度でおこるので詳細な検討が可能であり，この形成が茎葉形成を導びくと推測されている．そこでこの緑化部位形成に対するオーキシン類・サイトカイニン類の効果に関する詳細な検討は，イネカルスの茎葉形成の機構を考える際，重要である．予備実験の結果，イネカルスの継代を重ねるにつれ，各種器官の形成率の低下とともに，発生した植物体やその植物の種子には各種の異常が多くなることが認められた．イネカルスの緑化の葉緑素組成はイネの葉のそれと同一であった．イネの緑化部位形成はタバコカルスにおける分化と異なり，オーキシンとサイトカイニンの濃度比により単純に制御されるのではなく，これらの物質に対して複雑な対応を示した．この緑化部位形成にはオーキシンが必要であることを指摘した．イネカルスの緑化部位形成の特異性を示すため，イネとヒエのカルスの比較を行った．そのほか，緑化部位形成とこれに影響する各種因子との関連性について考察した．以上のイネカルスの緑化に関する基礎的知見はイネの葉の葉緑体の発生，発達，保持などの問題を解明する上で有用と考えられる．