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Effects of Plant Growth Regulators on the Response in Immature Wheat Embryo Culture

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The purpose of this study was to investigate the effects of 2,4-D, 6-furfurylamino purine (kinetin), and abscisic acid (ABA) on the response of cultured immature wheat (*Triticum aestivum* L.) embryos, by evaluating direct germination, callus growth, shoot regeneration and its development.

The reduction of 2, 4-D concentration or the addition of kinetin to callus induction media promoted direct germination of cultured embryos, whereas the addition of ABA suppressed it extremely. Callus growth was promoted by reducing 2, 4-D concentration or by adding ABA at lower concentrations, though these alterations did not affect the frequency of callus formation. Concentrations of 2, 4-D in callus induction media did not affect the frequency of subsequent shoot regeneration from calli; however, the regenerated shoots developed rapidly when calli were induced at lower 2, 4-D. The addition of kinetin or ABA significantly improved the shoot-forming ability of callus cultures, especially in less responsive cultivars.

Regenerative potential of callus cultures varies significantly depending on the donor cultivars or organs. Regeneration in wheat has been demonstrated for numerous cultivars by culturing immature embryos.[1-8] However, callus cultures of some cultivars produce few shoots,[4,5,9-13] and even attempts to induce callus from several other cultivars have failed.[4] Consistent shoot formation occurs for most cultivars when 2, 4-D is used as the auxin source.[3-5,11] However, no callus formation was observed when IAA or naphthalene acetic acid (NAA) was used as the only auxin source.[3] Also, it has been known that the optimum concentration of 2, 4-D for callus formation differs among cultivars, and regenerative potential varies among calli induced at different 2, 4-D concentrations.[14] Recently, with increased understanding of hormonal and nutritional requirement of various morphogenetic processes, further improvement has been achieved in wheat tissue culture. The frequency of embryogenic callus formation from immature wheat embryos increased by using the combination of 2, 4-D, NAA, and 6-benzyladenine (6-BA) rather than 2, 4-D only,[15] and pollen embryoid formation was promoted by ABA in wheat anther culture.[16,17] In the present study, we report that efficient shoot formation could be realized especially in less responsive cultivars by applying different hormonal combinations in callus induction medium.

Materials and Methods

Four cultivars, Chinese Spring (C. S.), Aobakomugi, Norin No. 61, and Nongda 146 were chosen for their response to tissue culture in the previous study.[18] Immature caryopses were taken from main spikes at 10 to 14 days postanthesis and were surface-sterilized first in 70% ethanol for 10 sec, then in 1.5% sodium hypochlorite solution with a drop of Tween 20 for 10 min, followed by several rinses with sterile distilled water. Ten embryos, which were selected for uniformity of about 1 mm in diam-

eter, were cultured on a disposable petri dish (100×15 mm) with the scutellum upwards. At least 30 embryos were cultured for each treatment. Petri dishes were sealed with Novix-II (Iwaki Glass Co. Ltd.) and incubated at 26±1℃ in the dark. After 3 weeks, frequencies of direct germination and callus formation, diameter and fresh weight of calli were assessed. The calli were transferred to regeneration medium and incubated under fluorescent illumination of about 2,000 lux (14 hr/day) at 26±1℃ for 4 more weeks to determine their shoot-forming ability.

The basal medium was the formula of Murashige and Skoog (MS) supplemented with 3% sucrose. The kinds and concentrations of growth regulators were modified as shown in **Tables 1-3**. All the media were adjusted to pH 5.8 and autoclaved for 15 min at 120℃.

Results

Frequencies of direct germination in cv. C. S., Aobakomugi, and Norin No. 61 on the control medium (2, 4-D, 2 mg/*l*) were 20.0, 0, and 1.9%, respectively (**Table 1**). Their frequencies increased to 38.9,

Table 1. Effects of 2, 4-D and kinetin (KN) in callus induction media on the response of cultured immature wheat embryos.

Cultivars	Conc. (mg/*l*)		Direct germination (%)[a]	Callus diameter (mm)[a] (Mean±SD)	Shoot regeneration (%)[b]
	2, 4-D	KN			
Chinese Spring	2	0 (Cont.)	20.0	5.1±0.3 bc	80.7
	2	0.25	25.0	5.3±0.4 cd	98.2
	2	0.5	35.3	5.3±0.3 cd	97.7
	2	1	39.6	5.0±0.4 ab	100.0
	2	2	54.9	4.8±0.4 a	97.8
	1	0	38.9	5.7±0.6 f	100.0
	1	0.25	54.7	5.6±0.4 ef	100.0
	1	0.5	64.7	5.4±0.3 de	100.0
	1	1	71.7	5.4±0.4 de	100.0
	1	2	73.6	4.9±0.4 ab	91.7
Aoba-komugi	2	0 (Cont.)	0	5.0±0.4 a	95.9
	2	0.25	2.0	5.3±0.4 ab	100.0
	2	0.5	8.0	5.5±0.4 bcd	94.4
	2	1	9.8	5.4±0.4 bc	100.0
	2	2	13.5	5.0±0.4 a	100.0
	1	0	11.8	6.4±0.6 e	97.9
	1	0.25	19.6	5.8±0.6 d	100.0
	1	0.5	25.9	5.7±0.5 cd	100.0
	1	1	26.9	5.6±0.5 bcd	100.0
	1	2	30.8	5.3±0.4 ab	100.0
Norin No. 61	2	0 (Cont.)	1.9	5.2±0.5 a	55.8
	2	0.25	5.7	5.4±0.5 ab	95.9
	2	0.5	7.8	5.2±0.5 a	97.9
	2	1	9.4	5.2±0.5 a	88.7
	2	2	12.0	5.4±0.5 ab	98.0
	1	0	5.6	5.7±0.7 b	57.1
	1	0.25	21.1	5.8±0.7 b	96.2
	1	0.5	21.2	5.5±0.7 ab	80.4
	1	1	47.9	5.7±0.6 b	96.0
	1	2	66.7	5.4±0.5 ab	91.1

Data in the same column followed by different letters are significantly different at 1% level of probability according to Duncan's multiple range test.

[a] After 3 weeks of culture on callus induction media.

[b] After 4 weeks of culture on regeneration media.

Cont.: control.

11.8, and 5.6%, respectively, when 2, 4-D was used at 1 mg/*l*. The addition of kinetin also promoted direct germination. The maximum frequencies were 73.6, 30.8, and 66.7% in the three cultivars, respectively. The concentration of either 2, 4-D or kinetin did not affect the frequencies of callus formation, which showed 100% for all the cultivars and treatments. In the three cultivars, mean diameters of calli were significantly longer when 2, 4-D was used at 1 mg/*l* than at 2 mg/*l*. The added kinetin promoted callus proliferation in medium containing 2 mg/*l* of 2, 4-D, but suppressed that in medium containing 1 mg/*l* of 2, 4-D.

In the responsive cultivars (C. S. and Aobakomugi), the frequencies of shoot regeneration were nearly 100% in all the treatments except the control of C. S. (80.7%). In a less responsive cultivar (Norin No. 61), however, the frequencies were low (55.8 and 57.1%) when the calli were induced on media

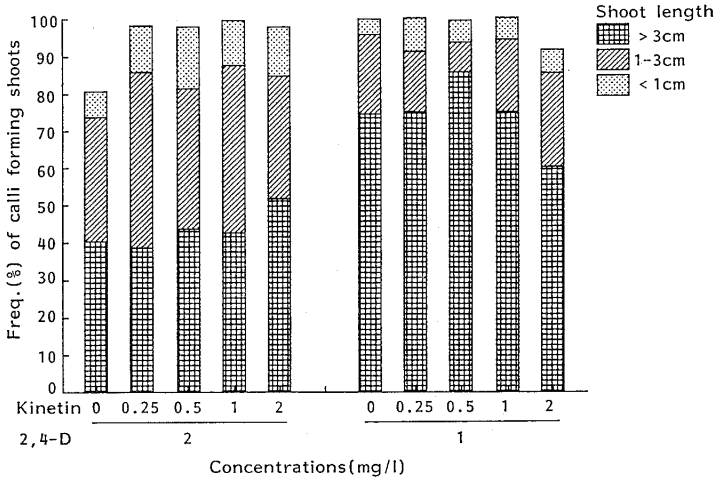

Fig. 1. Comparison of shoot development from calli formed on callus induction media containing 2, 4-D and kinetin with different combinations (cv. Chinese Spring).

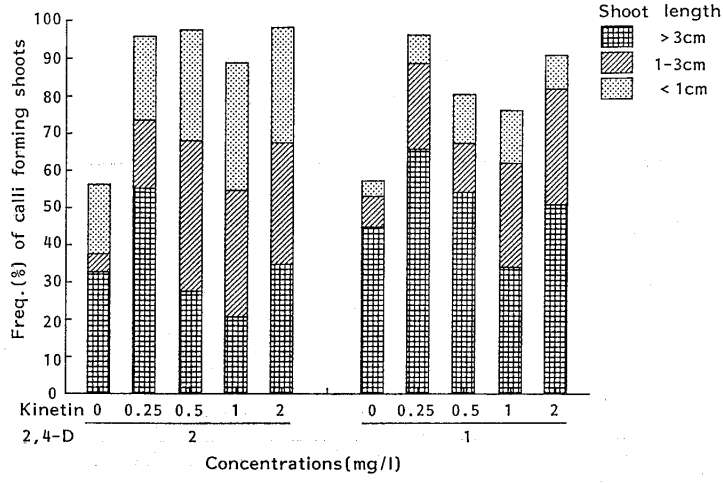

Fig. 2. Comparison of shoot development from calli formed on callus induction media containing 2, 4-D and kinetin with different combinations (cv. Norin No. 61).

containing only 2, 4-D at 2 or 1 mg/*l*. On the contrary, shoot regeneration was significantly promoted in this cultivar by adding kinetin to callus induction media even at low concentrations. This regeneration-promoting effect of kinetin was not influenced by concentrations of 2, 4-D.

Figures 1 and **2** show the developmental states of regenerated shoots from C. S. and Norin No. 61, respectively. In both cases, the frequencies of shoot regeneration were not affected by the concentration of 2, 4-D. However, the proportions of callus that produced shoots longer than 3 cm were obviously higher in the treatments of 2, 4-D at 1 mg/*l* than in those at 2 mg/*l*.

As shown in **Table 2**, direct germination of immature embryos was extremely suppressed by the addition of ABA even at 0.1 mg/*l*, and was suppressed completely when applying ABA at more than 0.4 mg/*l*. Frequencies of callus formation showed approximately 100% in almost all the treatments, being not affected by the addition of ABA. The calli proliferated rapidly in the presence of ABA at all the concentrations except 1.2 mg/*l*: The mean callus diameter on ABA-free medium (3.8 mm) increased up to 5.5 mm on the medium containing ABA at 0.4 mg/*l*. The mean fresh weights of calli were closely related to their diameters, that is, the calli gained in fresh weight as the ABA concentration increased in the range of 0.1–0.4 mg/*l*. The calli that were initiated on the medium containing 2 mg/*l* of 2, 4-D and 0.4 mg/*l* of ABA showed the maximum fresh weight of 78.8 mg. This quantity was about twice as much as that of calli formed on the medium containing 2, 4-D only. Moreover, subsequent shoot regeneration from these calli was significantly promoted. The proportion

Table 2. Effects of ABA in callus induction media on the response of cultured immature wheat embryo culture (cv. Nongda 146, grown in the field).

ABA* (mg/*l*)	Direct germination (%)[a]	Callus diameter (mm)[a]	Callus weight (mg)[a] (Mean±SD)	Shoot regeneration (%)[b]
0	42.9	3.8±0.5 a	41.9±0.5 a	53.7
0.1	5.6	4.3±0.7 a	50.1±3.2 ab	61.1
0.2	2.9	4.9±0.9 b	54.9±1.9 ab	67.6
0.4	0	5.5±1.0 c	78.8±5.8 c	93.3
0.8	0	4.8±0.7 b	61.2±1.6 b	78.4
1.2	0	3.8±0.5 a	43.1±1.3 a	51.4

* Concentration of 2, 4-D in callus induction media was 2 mg/*l*.
[a,b] and letters a, b, c: see Table 1.

Table 3. Effects of different combinations of 2, 4-D, KN, and ABA in callus induction media on the response of cultured immature wheat embryos (cv. Nongda 146, grown in Koitotron).

Conc. (mg/*l*)			Direct germination (%)[a]	Callus formation (%)[a]	Callus weight (mg)[a]	Shoot regeneration (%)[b]
2, 4-D	ABA	KN				
2	0	0	13.9	100	33.5	77.1
1	0	0	57.1	100	44.6	82.9
1	0	0.25	51.4	100	40.1	89.3
1	0	1	55.9	100	31.7	83.3
1	0.1	0	16.2	100	23.4	90.5
1	0.1	0.25	19.5	100	18.2	82.1
1	0.1	1	5.0	95.0	15.8	91.4
1	0.4	0	0	100	20.8	71.4
1	0.4	0.25	2.8	97.2	18.0	48.6
1	0.4	1	2.3	68.2	14.8	66.7

[a,b]: see Table 1.

of calli that formed shoot increased up to 93.3% by supplying them with 0.4 mg/*l* of ABA, while it decreased down to 51.4% when ABA was used at 1.2 mg/*l*.

In **Table 3**, effects of various combinations of 2, 4-D, kinetin, and ABA in the callus induction medium were investigated. The reduction of 2, 4-D concentration from 2 to 1 mg/*l* promoted callus growth and subsequent shoot formation. Contrarily, the addition of kinetin and/or ABA to medium containing 1 mg/*l* of 2, 4-D tended to suppress callus growth. The mean fresh weight of calli formed on the medium containing both 1 mg/*l* of 2, 4-D and 0.4 mg/*l* of ABA showed only 20.8 mg, which was less than half of that obtained on the medium containing only 2, 4-D at 1 mg/*l*. The addition of kinetin at 0.25–1 mg/*l* and/or ABA at 0.1 mg/*l* promoted shoot formation. However, the addition of ABA at 0.4 mg/*l* inhibited it irrespective of kinetin concentration.

Discussion

Plant growth regulators, especially auxin, are known to play a very important role in the process of callus induction and its proliferation in plant tissue culture. In immature wheat embryo culture, 2, 4-D is widely used at 2 mg/*l* for its effectiveness in producing calli.[1,3,12,19,20] However, the optimum concentrations for inducing regenerative calli differ according to kinds of auxin and plant materials. Callus growth has been promoted by reducing 2, 4-D level in mature embryo culture of wheat.[21] Carman *et al.*[22] have reported that 2, 4-D at higher than 2 mg/*l* inhibited the formation of embryoids in immature wheat embryo culture, and they proposed that the optimum 2, 4-D concentration to induce and maintain somatic embryogenesis was about 0.8 mg/*l*. Since the reduction of 2, 4-D concentration significantly promoted callus growth and improved the development of regenerated shoots in the present study, it is considered that a low level of 2, 4-D, which was enough to induce callus, is favorable not only for the growth of embryogenic calli but also for efficient shoot regeneration from them.

Although kinetin enhanced direct germination, it did not affect callus formation and subsequent shoot regeneration. Therefore, direct germination itself is considered to be inoffensive for obtaining regenerative calli. In the previous study, addition of kinetin and 6-BA to medium containing a low level of 2, 4-D (1 mg/*l*) suppressed the growth of calli originated from wheat root.[23] It is consistent with the results of this study. From these results, it can be said that a combination of cytokinin with a low level of 2, 4-D is undesirable for callus growth in wheat.

The significant improvement in shoot regeneration that we observed when calli were initiated on medium containing 2, 4-D (1–2 mg/*l*) plus kinetin (0.25–2 mg/*l*) is not unique. Carman *et al.*[22] have reported that kinetin added to medium containing auxin significantly increased the number of somatic embryoids in immature wheat embryo culture. Papenfuss *et al.*[20] have also indicated that the addition of kinetin to medium containing 2, 4-D improved the shoot-forming ability of wheat callus cultures. Besides, there are several other reports concerning the promotive effects of cytokinin on organogenesis from wheat cell cultures.[2,10,23–25]

Maddock *et al.*[11] have observed that the addition of 10% coconut milk (CM) to the callus induction medium significantly promoted subsequent shoot regeneration, and the same effect of CM has been identified in another study.[3] It is supposed that the promotive effects of additives such as CM on the regenerative potential of callus cultures may be largely due to unidentified cytokinin activity.[26] In the present study, many embryoid-like structures were observed during the early period of callus induction, especially in the presence of kinetin. From the above results, it is considered that exogenous cytokinin in callus induction medium plays an essential role in embryoid formation from cultures, because this is consistent with a possible requirement in early zygotic embryo initiation and development.[22] Thus, it is possible to say that the effect of kinetin indicated in this study originated in induction of embryoid formation in the early period of callus formation ; however, these *de novo*-formed immature embryoids may not develop into mature ones and precociously regenerated into vigorous shoots. This may be due to the excessively accumulated kinetin within calli during callus initiation.

Triplett and Quantrano[27] have shown that ABA in callus induction medium prevented direct germination of immature wheat embryos, but permitted substantial increase in embryo size, fresh and dry

weight, and protein accumulation. Carman[28] has reported that the addition of ABA reduced the incidence of abnormal embryoids. But, the morphologically improved embryoids formed on ABA-supplementing medium hardly germinated. Also, Shimada *et al.*[16,17] have reported that ABA in anther culture medium in wheat enhanced formation of pollen embryoids to some extent, but it did not promote their further development. Since approximately 90% of endogenous ABA in excised immature soybean embryos diffused into an ABA-free solution within 2 days,[29] it is considered that the onset of direct germination of cultured immature wheat embryos may be related to the loss of endogenous ABA. Embryoids that formed within wheat calli might fail to accumulate functionally active concentrations of endogenous ABA because of failure to synthesize adequate amounts, or because of concentration gradient diffusion from embryoids to an ABA-free medium, so that the embryoids precociously germinated.[3,22] We suppose that ABA in callus induction medium might make embryoids accumulate a proper amount of ABA or might offset the diffusion away from developing somatic embryos. So, the addition of ABA at lower concentrations ensured the development of embryoids to a proper extent, and they finally generated into vigorous shoots on the regeneration medium in this study. The results in Carman's study,[28] where the embryoids produced on ABA-containing medium hardly germinated whereas the desiccation significantly enhanced their germination, could be explained as follows. The concentration of ABA (0.5 mg/*l*) was too high compared with that of 2, 4-D (0.8 mg/*l*), so the embryoids might accumulate excess ABA, which could suppress their germination. The results (**Table 3**) of our study support this consideration. That is, the addition of ABA at 0.4 mg/*l* to medium containing 1 mg/*l* of 2, 4-D resulted in significantly poor shoot regeneration. Contrarily, the promoting effect of desiccation on normal germination of embryoids will be explained by the reduction of ABA amount in embryoids, because the level of ABA in wheat grains declines during desiccation.[30,31] It is suggested that the balance of 2, 4-D and ABA is essential for induction of embryogenic calli and efficient plant regeneration.

All the above facts emphasize the importance of evaluating the effects of different combinations of plant growth regulators on tissue culture response, especially for less responsive wheat cultivars.

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コムギの未熟胚培養における植物生長調整物質の効果

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コムギの未熟胚培養の効率化をはかるため，カルス誘導培地中の植物生長調整物質の改変が器官形成に及ぼす効果について検討した．未熟胚の直接発芽は 2, 4-D 濃度の減少とカイネチン濃度の増加に伴ってかなり促進されたが，ABA の添加によって著しく抑制された．いずれの植物生長調整物質添加もカルスの形成率にはほとんど影響しなかったが，2, 4-D の濃度を下げるか，低濃度の ABA を加えることによってカルスの増殖が促進された．一方，2, 4-D の濃度変化は再分化率には大きな影響を及ぼさなかったが，低濃度区での再分化植物体の発育を明らかに促進した．再分化能の高い品種では，再分化率に対するカイネチンの添加効果はみられなかったが，再分化能の低い品種では KN の添加によって再分化率が顕著に向上した．また，低濃度の ABA 添加によって旺盛な植物体再分化が認められた．