Effects of Various Phytohormones on Haploid Wheat Production in Wheat x Maize Crosses

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Abstract: The effects of phytohormones on the production of haploid wheat were examined in the intergeneric crosses between Japanese wheat cultivar (Triticum aestivum cv. Zenkojikomugi) and maize (Zea mays cv. Pioneer P80 Lisa). The detached wheat spikes pollinated with maize were cultured in a solution containing sucrose and sulfuric acid supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), indole-acetic acid, naphthalene acetic acid, kinetin or 6-benzylaminopurine at 0, 0.1, 1, 10, 100 or 1000 mg L⁻¹. Haploid embryos obtained were cultured on agarose-solidified B5 medium. The frequency of plant regeneration was significantly affected only by the treatment with 100 mg L⁻¹ 2,4-D. Thus, the detached spikes were cultured in the medium containing 2,4-D at 0, 25, 50, 75, 100, 125, 150, 175, 200 or 400 mg L⁻¹. The treatments with 50 mg L⁻¹ 2,4-D increased the embryo size, but the treatments with above 75 mg L⁻¹ 2,4-D inhibited the development of the embryo. The percentage of florets developing into haploid plants was increased by the treatment with 100 mg L⁻¹ 2,4-D. Therefore, the concentration of 2,4-D in the spikes treated with 2,4-D at 50, 100 and 150 mg L⁻¹ were measured by gas chromatography. The concentration of 2,4-D in the seed was increased to 9.24 ppm by the treatment with 100 mg L⁻¹ 2,4-D, a further increase of 2,4-D concentration in the medium having no effect. On the other hand, the concentration of 2,4-D in the glumes and rachis increased up to 12.72 and 41.55 ppm by the treatment with 100 and 150 mg L⁻¹ 2,4-D, respectively. The treatments with 2,4-D at a concentration higher than 100 mg L⁻¹ inhibited embryo development. The present results suggested that 2,4-D at the concentrations from 50 to 100 mg L⁻¹ would be optimum for haploid wheat production using maize.

Key words: 2,4-Dichlorophenoxyacetic acid, Haploid, Intergeneric cross, Phytohormone, Plant regeneration, Triticum aestivum, Zea mays.

Intergeneric crossing, wheat (Triticum aestivum L.) x Hordeum bulbosum L. (Barclay, 1975) and wheat x maize (Zea mays L.) crosses (Laurie and Bennett, 1988) have been used to produce haploid wheat. In the crosses of wheat x H. bulbosum, treatment with 2,4-dichlorophenoxyacetic acid (2,4-D) at a concentration lower than 100 mg L⁻¹ was effective for haploid wheat production although 2,4-D treatment did not suppress the cross-incompatibility gene(s), Kᵣ alleles of wheat (Inagaki, 1986). However, the action of Kᵣ alleles cannot prevent fertilization when maize is used as the male plant (Laurie and Bennett, 1987, 1988; Suenaga, 1990). In addition, the variations of agronomic characters of doubled haploids obtained from the crosses of wheat x maize were similar to those found in the crosses of wheat x H. bulbosum. Therefore, we used wheat x maize crosses in this study.

In the crosses of wheat x maize, 2,4-D treatment promoted seed setting and embryo formation (Laurie and Bennett, 1988; Suenaga and Nakajima, 1989; Riera-Lizarazu and Mujeeb-Kazi, 1990; Kisana et al., 1993), but the optimum concentration of 2,4-D for embryo differentiation and the production of haploid wheat have not been determined. In this study, we determined the optimum concentration of 2,4-D and other phytohormones on the embryo formation and haploid plant regeneration in the wheat x maize crosses.

Materials and Methods

An F₁ hybrid of maize, Pioneer P80 Lisa, which heads early in a greenhouse in winter and has a high capacity of haploid production in the crosses with wheat (Ushiyama et al., 1991), was used in this study as a pollen source. The Japanese wheat cultivar, Triticum aestivum cv. Zenkojikomugi, which showed a medium frequency of haploid production in the crosses of wheat x maize and wheat x Hordeum bulbosum (Ushiyama et al., 1990), was used as a female parent. Wheat and maize were seeded every other day from September

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Abbreviations: BA, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-acetic acid; NAA, naphthalene acetic acid.
Table 1. Frequencies of haploid production in the crosses of wheat cultivar Zenkojikomugi x maize cultivar Pioneer P80 Lisa treated with phytohormones at various concentrations.

| Phytohormone | Concentration (mg L⁻¹) | No. of florets pollinated | No. of seeded florets (%) | No. of florets with embryos formed (%) | No. of florets with haploid plantlets (%) |
|--------------|-------------------------|---------------------------|---------------------------|----------------------------------------|-------------------------------------------|
| Control      | 0.0                     | 286                       | 9 (3.1)                   | 1 (0.4)                                | 1 (0.4)                                   |
| 2,4-D        | 0.1                     | 234                       | 10 (4.3)                  | 1 (0.4)                                | 1 (0.4)                                   |
| 2,4-D        | 1.0                     | 254                       | 6 (2.4)                   | 2 (0.8)                                | 1 (0.4)                                   |
| 2,4-D        | 10.0                    | 244                       | 37 (15.2)*                | 7 (2.9)                                | 1 (0.4)                                   |
| 2,4-D        | 100.0                   | 222                       | 162 (73.0)***             | 48 (21.6)***                           | 19 (8.6)***                               |
| 2,4-D        | 1000.0                  | 214                       | 0 (0.0)**                 | -                                      | -                                         |
| NAA          | 0.1                     | 222                       | 0 (0.0)*                  | -                                      | -                                         |
| NAA          | 1.0                     | 232                       | 0 (0.0)*                  | -                                      | -                                         |
| NAA          | 10.0                    | 224                       | 0 (0.0)*                  | -                                      | -                                         |
| NAA          | 100.0                   | 224                       | 2 (0.9)                   | 0 (0.0)                                | -                                         |
| NAA          | 1000.0                  | 204                       | 34 (16.7)                 | 7 (3.4)                                | 5 (2.5)                                   |
| IAA          | 0.1                     | 240                       | 2 (0.8)                   | 0 (0.0)                                | -                                         |
| IAA          | 1.0                     | 262                       | 5 (1.9)                   | 3 (1.1)                                | 1 (0.4)                                   |
| IAA          | 10.0                    | 228                       | 2 (0.9)                   | 1 (0.4)                                | 0 (0.0)                                   |
| IAA          | 100.0                   | 228                       | 9 (3.9)                   | 2 (0.9)                                | 0 (0.0)                                   |
| Kinetin      | 0.1                     | 226                       | 3 (1.3)                   | 1 (0.4)                                | 1 (0.4)                                   |
| Kinetin      | 1.0                     | 220                       | 4 (1.8)                   | 2 (0.9)                                | 0 (0.0)                                   |
| Kinetin      | 10.0                    | 220                       | 4 (1.8)                   | 2 (0.9)                                | 2 (0.9)                                   |
| Kinetin      | 100.0                   | 218                       | 2 (0.9)                   | 0 (0.0)                                | -                                         |
| BA           | 0.1                     | 230                       | 1 (0.4)*                  | 0 (0.0)                                | -                                         |
| BA           | 1.0                     | 236                       | 3 (1.3)                   | 1 (0.4)                                | 1 (0.4)                                   |
| BA           | 10.0                    | 264                       | 4 (1.5)                   | 1 (0.4)                                | 1 (0.4)                                   |
| BA           | 100.0                   | 300                       | 0 (0.0)**                 | -                                      | -                                         |
| BA           | 1000.0                  | 248                       | 0 (0.0)**                 | -                                      | -                                         |

Controls indicated that detached spikes were cultured in the medium without phytohormone. * , ** and *** indicates significant difference from the control at 5, 1 and 0.1% levels probability, respectively, by t-test with arcsine transformed data.

To February. The maize seedlings were grown in a greenhouse kept at the minimum temperature of 20°C. For haploid wheat cultivation, we used intergeneric crossing and embryo culture followed by the bulbosum system of Ushiyama et al. (2006). The experiment to determine useful phytohormones was carried out in
November and December 1990. After pollination, detached spikes were cultured in the solution, containing 10 mg L\(^{-1}\) ethanol, 0.05% sulfurous acid, 40 g L\(^{-1}\) sucrose and various phytohormones, i.e., 2,4-D, naphthalene acetic acid (NAA), indole acetic acid (IAA), kinetin and 6-benzylaminopurine (BA) at various concentrations. Unpollinated spikes were also cultured in the above solution excluding the solutions containing IAA, kinetin and BA. The numbers of seeded florets, florets with embryos formed and florets with regenerated plantlets were counted for every spike. These numbers were compared between treated and untreated plots by the t-test.

To determine the optimum concentration of phytohormones for the haploid production, we conducted the following experiment twice in January 1991. The numbers of seeded florets, florets with embryos and plantlets regenerated per embryo and per floret were counted. These data were transformed by arcsin square root transformation and subjected to analysis of variance. The mean length of embryos was measured two weeks after pollination and subjected to analysis of variance.

The concentration of 2,4-D in the fresh weight of spikes was measured (Goto and Kato, 1980) by gas chromatography (Shimadzu GC-8A) in March 1991. Five spikes were collected after each treatment with 2,4-D and divided into two parts, i.e., seeds and the other parts (glumes and rachis). The data were transformed and subjected to analysis of variance.

### Results

1. **The effect of phytohormone on haploid wheat production**

Table 1 shows the percentages of seeded florets, florets with embryos formed and florets from which haploid plants developed in the pollinated spikes of the crosses of wheat x maize treated with 2,4-D, NAA, IAA, kinetin and BA at various concentrations. At a concentration of 10 and 100 mg L\(^{-1}\) 2,4-D significantly increased the percentage of seeded florets to 15.2% and 73.0%, respectively (3.1% in the control). The percentage of seeded florets after treatment with other phytohormones were not higher than that in the untreated control. The percentages of florets with embryos formed and that with haploid plants developed, significantly increased by the treatment with 2,4-D at 100 mg L\(^{-1}\) (21.6% and 8.6% respectively) in comparison with the control (0.3% and 0.3% respectively). In the spikes without pollination, 2,4-D treatments at 10 and 100 mg L\(^{-1}\) significantly increased the percentage of seeded florets (22.8% and 55.6%), compared with untreated control (0.0%). Spikes treated with 1000 mg L\(^{-1}\) NAA set some seeds but the difference of the percentage of seeded florets was not significantly different from that in the control. However, seeds formed in the unpollinated florets had no embryo. This indicates that the treatment with various phytohormones induces parthenogenesis.

### Table 2

Percentages of florets that formed seeds, embryos and haploid plantlets in the crosses of wheat cultivar Zenkojikomugi x maize cultivar Pioneer P801 treated with 2,4-D at various concentrations.

| Concentration of 2,4-D mg L\(^{-1}\) | No. of florets pollinated (%) | No. of seeded florets (%)\(^{1}\) | No. of florets with embryos formed (%)\(^{2}\) | Mean length of embryos mm | No. of florets with haploid plantlets (%)\(^{3}\) |
|--------------------------------|-----------------------------|-----------------------------|---------------------------------|-----------------------------|--------------------------------|
| 0                             | 157                         | 2 (1.3)g                    | 1 (0.6)cd                       | −                           | 1 (100.0)                      |
| 25                            | 166                         | 58 (34.9)cf                 | 15 (9.0)ab                      | 1.19bc                      | 10 (66.7)                      |
| 50                            | 216                         | 109 (50.5)cd                | 33 (15.3)a                      | 1.59a                       | 22 (66.7)                      |
| 75                            | 244                         | 147 (60.2)cd                | 32 (13.1)a                      | 1.34b                       | 20 (62.5)                      |
| 100                           | 402                         | 241 (60.0)bc                | 79 (19.7)a                      | 1.08c                       | 40 (50.6)                      |
| 125                           | 208                         | 180 (86.5)a                 | 34 (16.3)a                      | 1.02c                       | 8 (23.5)                       |
| 150                           | 190                         | 159 (83.7)ab                | 26 (13.7)a                      | 1.07c                       | 11 (42.3)                      |
| 175                           | 200                         | 77 (38.5)de                 | 24 (12.0)ab                     | 1.06c                       | 2 (8.3)                       |
| 200                           | 244                         | 35 (14.3)bc                 | 7 (2.9)bc                       | 0.96c                       | 2 (28.6)                      |
| 400                           | 230                         | 0 (0.0)g                    | 0 (0.0)c                        | −                           | −                            |

\(^{1}\) indicates frequency of embryo formation per floret.

\(^{2}\) indicates percentage of embryos developing into haploid plants.

\(^{3}\) indicates percentage of florets developing into haploid plants.

\(^{4}\) Any two means having the same letter in each column are not significantly different at the 5% probability level by Duncan’s multiple range test with arcsine transformed data.
2. The optimum concentration of 2,4-D for haploid production
Table 2 shows the frequency of haploid production in the crosses of Zenkojikomugi x Pioneer P80 Lisa treated with 2,4-D at various concentrations. Table 3 shows the results of the analyses of variance of these data. Differences among 2,4-D concentrations in the percentages of seeded florets, florets with embryos formed, and florets with haploid plants developed, were significant at the 1 or 0.1% level (Table 3). The percentages of seeded florets in the spikes treated with 2,4-D at the concentrations of 125 and 150 mg L⁻¹, were 86.5% and 83.7%, respectively, and significantly higher \((p < 0.05)\) than those at the other concentrations (0.0% to 60.2%) by Duncan’s multiple range test. The percentage of seeded florets increased with increasing concentration of 2,4-D up to 125 mg L⁻¹ 2,4-D, but decreased at higher concentrations. The percentage of florets with embryos formed was significantly increased by 2,4-D at the concentration of 25 to 200 mg L⁻¹. Analysis of variance for embryo size at two weeks after pollination showed that the embryo size was significantly influenced by the concentration of 2,4-D at the 0.1% level. The mean length of the embryo in the spikes treated with at 50 mg L⁻¹ 2,4-D was longer than that at the other concentrations. The percentage of embryos developed into haploid plants was not significantly influenced by the concentration of 2,4-D (Table 3). The percentages of florets with haploid plants developed was significantly influenced by the concentration of 2,4-D (Table 2), and was the highest (10.2%) at 50 mg L⁻¹. The treatments with 2,4-D at the concentrations from 25 to 150 mg L⁻¹ (3.8% to 10.2%) significantly increased haploid production compared with the untreated control and the treatments at other concentrations of 2,4-D (0% to 1.0%).

3. Concentration of 2,4-D in the spikes
Table 4 shows the concentrations of 2,4-D in the seed and glumes + rachis on the spikes treated with 50, 100 and 150 mg L⁻¹. Table 5 shows the results of the analysis of variance of the data. The concentration of 2,4-D in the spikes significantly differed both with the 2,4-D concentration in the medium for spikelet culture and among the parts of the spikes. The concentration of 2,4-D in the seed on the spikes treated with 50 mg L⁻¹ 2,4-D (1.15 ppm) was significantly lower than that treated with 100 and 150 mg L⁻¹ 2,4-D (9.24 and 6.55 ppm, respectively). However, the concentration of 2,4-D in the glumes and rachides on the spikes treated with 2,4-D 150 mg L⁻¹ (41.55 ppm), was significantly higher than that treated with 50 mg L⁻¹ 2,4-D (5.53 ppm) and 100 mg L⁻¹ 2,4-D (12.72 ppm).

Discussion
The results showed that 2,4-D was more useful for haploid wheat production than either NAA, IAA, kinetin or BA. In the crosses of wheat x maize, the high embryo recovery by 2,4-D treatment was already reported (Laurie and Bennett, 1988; Suenaga and Nakajima, 1989; Riera-Lizarazu and Mujeeb-Kazi, 1990;...
Kisana et al., 1993). In addition, the present results indicated that 2,4-D and NAA, namely synthetic auxin, might be more effective for haploid wheat production than cytokinin. However, in the previous experiments using wheat x maize crosses, the promotive effect of 2,4-D treatment on the plant regeneration from haploid wheat embryo was not clear. In this study, the frequency of plant regeneration was significantly increased by the treatment with 100 mg L\(^{-1}\) 2,4-D. The embryo development was inhibited by the treatment with over 50 mg L\(^{-1}\) 2,4-D. In the crosses of wheat x H. bulbosum, similar results was observed by Ushiyama et al. (2006), who pointed out that the treatment with 2,4-D at the concentration of 100 mg L\(^{-1}\) or less was effective for haploid wheat production while it reduced embryos sizes and arrested embryo development into plant. However, the mechanism of the effect of 2,4-D was not elucidated. The treatment with 2,4-D at 50 mg L\(^{-1}\) was most effective for the development of the embryo into the plant and the increase of embryo size, and that at 100 mg L\(^{-1}\) for haploid production. The optimum concentration of 2,4-D for seed setting was 125~150 mg L\(^{-1}\). We measured the concentration of 2,4-D in the spike treated with 2,4-D at different concentrations. The increase of the concentration of 2,4-D beyond 100 mg L\(^{-1}\) up to 150 mg L\(^{-1}\), did not increase the concentration of 2,4-D in the seed, but increased the concentration of 2,4-D in the glumes and rachis. Maddock et al. (1983) reported that 1.0 mg L\(^{-1}\) 2,4-D was optimum for induction of shoot development in cultured immature embryos, and 5 mg L\(^{-1}\) 2,4-D decreased the percentage of shoot-forming calli, though it increased callus growth. In this study, the concentration of 2,4-D in the seed on the spikes treated with 50 mg L\(^{-1}\) 2,4-D was 1.15 ppm, and embryo development was increased under this condition. 2,4-D in the seed at a concentration higher than 6.55 ppm may inhibit the normal activity of the embryo though it increased seed setting. Riera-Lizarazu and Mujeeb-Kazi (1990) pointed out that the rate of embryo recovery was higher in the detached spikes cultured in the medium containing 2,4-D than in the tiller injected with 2,4-D solution and in the floret sprayed with 2,4-D. 2,4-D presumably stimulated the development in immature florets development a few days after pollination. The concentrations of 2,4-D in the seeds reached 5ppm earlier on the spikes treated with 150 mg L\(^{-1}\) 2,4-D than with 100 mg L\(^{-1}\) 2,4-D. Some seeds without embryo were also set by the treatments with 2,4-D (10 and 100 mg L\(^{-1}\)) and NAA (1000 mg L\(^{-1}\)) in emasculated florets. The frequencies of seed setting in the unpollinated florets treated with these phytohormones paralleled those in the pollinated spikes. Marshall et al. (1983) reported that the application of a single drop of 80 mg L\(^{-1}\) or 160 mg L\(^{-1}\) 2,4-D solution to them increased the size of seeds, and Inagaki (1986) reported that 70.2% of the unpollinated florets set seeds without embryos when the culms were injected with 1000 ppm 2,4-D. Falk and Kashia (1982) reported that the unpollinated spikes sprayed with 0.1 and 1.0 mg L\(^{-1}\) IAA did not set seeds. Ushiyama et al. (2006) investigated the influence of various concentrations of 2,4-D on the production with wheat haploid and pointed out that the treatment with 2,4-D at 125 to 175 mg L\(^{-1}\) increased the percentage of the seed setting, but reduced the formation and development of the embryo. Therefore, it appears that the promoting effect of 2,4-D and NAA on seed setting is not reflected in fertilization and embryo development. The percentage of seeded florets without embryos formed was low, but that of florets with embryos formed and developed was high in the spikes treated with 2,4-D at the optimum concentration. It is, therefore, concluded that the treatment with 2,4-D at 50 to 100 mg L\(^{-1}\) was useful to produce haploid wheat plant in the crosses of Zenkohikomugi x maize. The embryo development may depend on the concentration of 2,4-D in the seeds.

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