Effect of 2,4-Dichlorophenoxyacetic Acid on the Efficiency of Wheat Haploid Production by the *Hordeum bulbosum* Method

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Abstract: Intergeneric crosses between Japanese wheat cultivars (*Triticum aestivum* L. cv. Nishikazekomugi and Zenkojikomugi) and a tetraploid wild barley, *Hordeum bulbosum* L., were used to examine the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on the formation of wheat haploid embryo and its development into plantlets. The detached wheat spikes with florets pollinated with *H. bulbosum* were cultured for 14 days in a sucrose and sulfurous acid solution to which 2,4-D was added at the concentrations of 0, 25, 50, 75, 100, 125, 150, and 175 mg L$^{-1}$. The percentages of florets with seeds set and with embryos formed were increased by increasing the concentration of 2,4-D up to 100 mg L$^{-1}$. Fourteen days after pollination, embryos (haploid) were isolated from the seed and cultured on agarose-solidified B5 medium. Embryo size tended to decrease as the concentration of 2,4-D increased, but the larger embryos tended to have higher ability to develop into haploid plants. The percentage of florets from which haploid plantlets were developed by embryo culture was slightly increased by the treatment of the spikes with 25-100 mg L$^{-1}$ 2,4-D, but significantly reduced by 125-175 mg L$^{-1}$ 2,4-D. It is suggested that treatment with 2,4-D at 25-100 mg L$^{-1}$ would be effective for haploid wheat production by of *H. bulbosum* method.

Key words: 2,4-dichlorophenoxyacetic acid, Embryo size, Haploid, *Hordeum bulbosum*, Intergeneric cross, Plant regeneration, *Triticum aestivum*, Wheat.

For generation of haploid wheat plants, the wheat plants have been pollinated with some Gramineae species: *Hordeum bulbosum* (Barclay, 1975), maize (*Zea mays* L.) (Laurie and Bennett, 1988), pearl millet (*Pennisetum americanum* L.) (Ahmad and Comeau, 1990), teosinte (*Zea mays* ssp. *mexicana*) (Ushiyama et al., 1991) and sorghum (*Sorghum bicolor* L.) (Ohkawa et al., 1992). Treatment with 2,4-D at a concentration of 100 mg L$^{-1}$ increased embryo formation in crosses of wheat x *H. bulbosum* (Inagaki, 1986) and wheat x maize (Suenaga, 1990). However, the concentration of 2,4-D suitable for embryo development and the production of haploid wheat has not been determined. The action of *Kr* alleles cannot prevent embryo formation in the absence of 2,4-D treatment when maize is used as the male parent (Laurie and Bennett, 1987, 1988; Suenaga and Nakajima, 1989). On the other hand, in crosses of wheat x *H. bulbosum*, haploid embryos were formed and developed into plants in the absence of 2,4-D although few haploid plants if any were obtained from the crosses between wheat cultivars having *Kr* allele(s) (Snape et al., 1979, 1980; Sitch et al., 1985). In this report, we describe the effects of 2,4-D on the growth of the haploid embryos and their development into plantlets in crosses of wheat x *H. bulbosum*.

Materials and Methods

One clone of tetraploid *H. bulbosum* (2n=4X=28), that was selected from the Nagano Agricultural Experiment Station collection nursery by Ushiyama, was used in this study as a pollen source. Two hexaploid Japanese wheat cultivars (*Triticum aestivum* L. 2n=6X=42), Nishikazekomugi and Zenkojikomugi, were used as female parents. When these two wheat cultivars were crossed with *H. bulbosum* in the absence of treatment with 2,4-D, the frequencies of plant development from the embryos markedly differed with the cultivar; 19.0% in Nishikazekomugi and 2.8% in Zenkojikomugi (Ushiyama et al., 1990). *H. bulbosum* bulbs were transplanted in September and the seedlings were grown for more than one month in a cold chamber kept at 5-10°C under an 8-hour photoperiod as a clone bank. Thereafter they were vernalized in a greenhouse kept at 5-10°C under a 12-h photoperiod under artificial light for eight weeks. Wheat seeds were seeded every other day.
from October to February and placed in the above greenhouse for six weeks for vernalization. These plant materials after the vernalization were grown in a greenhouse kept at a minimum temperature of 15ºC under natural day length conditions from November to March and flowering occurred after 4 to 5 weeks.

Two or three days before anthesis, the apical and basal spikelets of wheat, and all florets except for the two outermost florets on the remaining spikelets were removed, and the remaining florets were emasculated. For each treatment with 2,4-D, in each cross, ten to fifteen spikes (approximately 200 florets) per replication were pollinated with fresh pollen of tetraploid *H. bulbosum*. These pollinated spikes with stems were cut at the middle of the third internode and covered with a vinyl bag. Leaf blades were removed. The basal part of such detached spike was put into a conical beaker filled culture solution described below. Spikes were cultured in the solution containing 10.0 mL L⁻¹ ethanol, 0.8 mL L⁻¹ sulfurous acid (about 6% assay), 40 g L⁻¹ sucrose and 2,4-D at 0, 25, 50, 75, 100, 125, 150, and 175 mg L⁻¹. One day after pollination, the spikes were sprayed with 75 mg L⁻¹ gibberellic acid solution and transferred into a growth chamber controlled at 22ºC under a 12-h photoperiod under artificial light. The vinyl bag was replaced by a paper bag at this time.

Fourteen days after pollination, seeds were collected and surface-sterilized in a test tube with 1% sodium hypochlorite solution for 10 minutes, and then with 80% ethyl alcohol solution for one minute. The embryos were isolated from the caryopses that grew over two third of the glume length. The each embryo was transferred onto agarose-solidified B5 medium (Gamborg et al., 1968) in a 20mm glass test tube. The embryos were incubated in the growth room (10-h photoperiod under fluorescent light; day/night temperature, 25/18 1ºC).

Intergeneric crosses were carried out three times (the first in January 1990, the second in March 1990 and the third in January 1991). Embryo sizes (maximum length) were measured in the second and the third experiments. The number of plants developed was counted one month after embryo rescue.

The frequency of seed setting, embryo formation, and the percentages of embryos and florets developing into plantlets were measured in every replication. All the percentages were calculated by arcsine square root transformation. The data were subjected to analysis of variance as the general two-factor experiment for every parameter and subjected to Duncan’s multiple range tests using the result shown by the analysis of variance.

| Wheat cultivar | Concentration of 2,4-D (mg L⁻¹) | No. of florets pollinated (%) | No. of seeded florets (%) | No. of florets with embryos formed (%) | No. of embryos developing into haploid plants (%) | Percentage of florets from which haploid plants developed (%) |
|---------------|--------------------------------|-----------------------------|-------------------------|--------------------------------------|-----------------------------------------------|----------------------------------------------------------|
| Nishikazekomugi | 0 | 734 | 199 (29.2) de | 143 (21.1) | 121 (85.9) a | (17.8) abc |
| | 5 | 800 | 312 (44.9) bcd | 206 (28.6) | 160 (79.7) ab | (23.0) ab |
| | 0 | 620 | 371 (58.8) abc | 176 (28.2) | 141 (81.7) ab | (22.7) ab |
| | 75 | 466 | 315 (67.5) ab | 141 (31.8) | 110 (77.1) ab | (24.6) ab |
| | 100 | 540 | 393 (72.6) ab | 221 (42.5) | 152 (67.9) abc | (29.4) a |
| | 125 | 544 | 426 (77.8) a | 174 (31.9) | 65 (38.7) de | (12.0) bcd |
| | 150 | 540 | 425 (78.9) a | 146 (26.0) | 34 (22.4) cf | (6.0) def |
| | 175 | 496 | 396 (76.8) a | 182 (33.8) | 22 (8.9) f | (3.7) ef |
| Zenkojikomugi | 0 | 620 | 70 (11.1) e | 51 (8.0) | 33 (61.8) bcd | (5.1) def |
| | 25 | 478 | 187 (38.5) cd | 72 (15.8) | 40 (53.2) de | (8.9) cde |
| | 50 | 574 | 348 (61.0) abc | 89 (15.4) | 40 (43.7) cd | (6.9) def |
| | 75 | 578 | 365 (62.9) abc | 96 (16.6) | 49 (53.4) cd | (8.6) cde |
| | 100 | 812 | 617 (75.7) a | 168 (20.8) | 68 (41.0) de | (8.4) cde |
| | 125 | 688 | 507 (74.0) a | 84 (11.9) | 12 (14.1) f | (1.7) ef |
| | 150 | 740 | 586 (78.8) a | 83 (13.1) | 9 (12.1) f | (1.4) f |
| | 175 | 672 | 498 (75.2) a | 97 (15.4) | 8 (7.9) f | (1.1) f |

1) Percentages followed by same letters in each column are not significantly different at the 5% probability level by Duncan’s multiple range test with arcsine square root transformed data.
The size of the embryo at two weeks after pollination was determined and classified according to the capability of developing into a plantlet into two categories, i.e., embryos developing and those not developing into plants. Mean lengths of embryos were calculated as the average of the two replications. Embryo sizes in each category and each cultivar were subjected to one-way analysis of variance and subjected to Duncan’s multiple range tests using the result of the analysis of variance. The difference between the mean lengths of embryos in the two cultivars was analyzed by the t-test.

Results

Table 1 shows the effects of the treatment with 2,4-D on haploid production in the two wheat cultivars, Nishikazekomugi and Zenkojikomugi, crossed with *H. bulbosum*, Table 2 shows the results of the analyses of variance.

1. Frequency of seed setting

In the absence of 2,4-D, the percentage of seed setting in the pollinated florets was 29.1 and 11.1% in Nishikazekomugi and Zenkojikomugi, respectively, and 2,4-D treatment increased the percentage (Table 1). The analysis of variance showed a significant difference at 0.1% level among the treatments with 2,4-D at various concentrations, but no significant difference between the wheat genotypes (Table 2). Analysis using Duncan’s multiple range tests showed that the percentage of seed setting was significantly increased by the treatment with 2,4-D at 50 mg L^{-1} or higher concentrations in both cultivars (Table 1). In the cross of Nishikazekomugi x *H. bulbosum*, the percentage of seed setting was increased to 77.8% by the treatment with 125 mg L^{-1} 2,4-D, but further increase in 2,4-D concentration had only a slight effect. In Zenkojikomugi x *H. bulbosum*, the percentage of seed setting was increased to 75.7% by the treatment with 2,4-D at 100 mg L^{-1} 2,4-D, and further increase in the concentration hardly increased the percentage.

2. Frequency of embryo formation

The percentage of florets that formed embryos was significantly higher in Nishikazekomugi than in Zenkojikomugi (*p* < 0.001) (Tables 1 and 2). On the other hand, the difference in the frequencies of embryo formation among the 2,4-D treatments at various concentrations was not significant. The highest percentage of embryo formation was observed in the florets on the spikes treated with 100 mg L^{-1} 2,4-D in both cultivars (42.5% in Nishikazekomugi and 20.8% in Zenkojikomugi) and the percentage tended to decrease at higher concentrations of 2,4-D (Table 1).

3. Frequency of plant development

Significant variation at the 0.1% level in the percentage of embryos developing into plantlets was observed among the 2,4-D treatments at various concentrations (Table 2). The percentage in the absence of 2,4-D was 85.9% in Nishikazekomugi and 61.8% in Zenkojikomugi, and the percentage tended to decrease as the concentration of 2,4-D increased (Table 1). When the concentration of 2,4-D was increased from 100 to 175 mg L^{-1}, the percentage of embryos developing into plantlets markedly decreased from 67.9% to 8.9% in Nishikazekomugi and from 41.0% to 7.9% in Zenkojikomugi.

The percentage of florets developing into plantlets significantly varied with the concentration of 2,4-D added, at 0.1% level (Table 2). The percentage was slightly increased by the treatment with 100 mg L^{-1} 2,4-D, from 17.8 to 29.4% in Nishikazekomugi and from 5.1 to 8.4% in Zenkojikomugi. However, the treatment with 2,4-D at 125 mg L^{-1} or higher concentrations significantly decreased the percentage of florets developing into plantlets.

The percentages of embryos and florets developing into plantlets were significantly higher in Nishikazekomugi than in Zenkojikomugi (*p* < 0.001) (Table 2). Duncan’s multiple range tests showed that the percentage of embryos or florets developing into plantlets was significantly higher in Nishikazekomugi than in Zenkojikomugi after the treatment with 2,4-D.
at the concentrations lower than 125 mg L\(^{-1}\).

4. **Embryo size**

Table 3 shows the relationship between the size and the ability to develop into plantlets of embryos. Analysis of variance for the size of embryo developing and not developing into plantlets in Nishikazekomugi showed significant difference among 2,4-D treatments at various concentrations (M.S. = 22.66, \(p < 0.001\) and M.S.=5.40, \(p < 0.001\)). There were also significant differences in embryo size among 2,4-D treatments in Zenkojikomugi (for embryos developing into plants, M.S.=3.24(7df), V.R.=24.96, \(p < 0.001\). Analysis of variance for the size of embryos developing into plants: M.S.=3.89, \(p < 0.01\), and for those not developing into plants, M.S.=10.63, \(p < 0.01\). Duncan’s multiple range tests showed that the sizes of embryos developing into plantlets in the fl orets treated with 2,4-D at the concentration above 50 mg L\(^{-1}\) in Nishikazekomugi and above 75 mg L\(^{-1}\) in Zenkojikomugi were smaller (\(p < 0.05\)) than those without 2,4-D treatment; 1.87 mm and 1.61 mm in Nishikazekomugi and Zenkojikomugi, respectively. The treatment with 2,4-D at a concentration above 75 mg L\(^{-1}\) also reduced the sizes of embryos not developing into plants.

In Nishikazekomugi, the size of embryos developing into plants was significantly larger than that of embryos not developing into plants except for the fl orets on the spikes treated with 75 mg L\(^{-1}\) 2,4-D. On the other hand, in Zenkojikomugi, there was no difference in size between embryos developing and not developing into plants, except for those formed on the spikes treated with 0 (control) and 100 mg L\(^{-1}\) 2,4-D.

**Discussion**

Marshall et al., (1983) indicated that application of 2,4-D to ovules increased ovule size in wheat (cv. Cook). Inagaki (1986) showed that the injection of 100 mg L\(^{-1}\) 2,4-D into the culm internode significantly increased the frequencies of seed setting in the crosses of two wheat cultivars (Fukuhokomugi and Haruhikari) with *H. bulbosum*. In the present study, the treatment with 2,4-D at 50 mg L\(^{-1}\) to 175 mg L\(^{-1}\) increased the percentage of seed setting. Thus, it is clear that seed setting is promoted by treating spikes with 2,4-D. The two wheat cultivars, Nishikazekomugi and Zenkojikomugi, used in this work did not show significant differences in the frequency of seed setting.
at all 2,4-D concentrations tested. On the other hand, Inagaki (1986) indicated that the frequency of seed setting in Fukuhokomugi (87.9%) was much higher than that in Haruhikari (2.5%) when crossed with H. bulbosum. This discrepancy may be due to the difference in the method of treatment with 2,4-D and/or in the wheat cultivars.

Inagaki (1986) reported that the treatment with 100 mg L\(^{-1}\) 2,4-D significantly increased the frequency of embryo formation in the cross of a crossable cultivar Fukuhokomugi with H. bulbosum, but only slightly in the cross of non-crossable cultivar Haruhikari with H. bulbosum. In the present experiment, although there were no significant differences among the frequencies of embryo formation in the florets treated with various concentrations of 2,4-D, the highest frequency was observed by the treatment with 2,4-D at 100 mg L\(^{-1}\). However, the treatment with 2,4-D in this study did not reduce the difference between the two wheat cultivars crossed with H. bulbosum in the percentage of embryos developing into plantlets. Therefore, it is likely that embryo formation was stimulated by 2,4-D treatment through increased number of seeds. In addition, Suenaga (1990) showed that treatment with 10 and 1,000 mg L\(^{-1}\) 2,4-D affected the frequency of embryo formation only slightly but that with 100 mg L\(^{-1}\) 2,4-D greatly increased the frequency, in wheat x maize crosses. Furusho et al. (1991) reported that 2,4-D at 75 mg L\(^{-1}\) was most effective for obtaining the embryos and plantlets, and that of 50 mg L\(^{-1}\) and 100 mg L\(^{-1}\) was less effective. In previous reports, it was not stated whether or not the treatment with 2,4-D had significant effect on the development of haploid wheat embryos into plantlets. In the present work, however, the treatment with 2,4-D at higher than 125 mg L\(^{-1}\) clearly reduced the percentage of embryos developing into plantlets, although the effect of 2,4-D at a concentration below 100 mg L\(^{-1}\) was not significant.

Inagaki (1985) reported that the optimum size of wheat haploid embryo for plant development was 1.4 mm in length (at two weeks after pollination), and 96.6% of such embryos developed into plantlets at three weeks after pollination in the absence of 2,4-D. When the embryo size became larger (2.28 mm), the frequency of plant development was lower (16.6%). Sitch and Snape (1986) reported that embryos developing into haploid plantlets in the absence of 2,4D were 1.19 mm in length on the average, and those failed to develop into plantlets and those formed calli were 0.61 and 0.67 mm in mean length, respectively. In the present investigation, the embryos including those capable and incapable of developing into plantlets had a mean length between 1.87 and 0.93 mm, which were smaller than the overgrowing embryos (2.28 mm) (Inagaki, 1985) and larger than the embryos immature for the embryo culture (0.61 or 0.67 mm) (Sitch and Snape, 1986). Therefore, the treatment with 2,4-D is considered to inhibit the growth of embryos.

In conclusion, above three factors (frequency of seed setting, embryo formation and development of embryo into plantlets) play important roles in the production of haploid plants. The examination of these factors is indispensable to plan the doubled haploid production for wheat breeding. Although it is not known whether or not 2,4-D treatment has an effect on the crossabilities in intergeneric crosses between wheat and H. bulbosum, the present study revealed that 2,4-D treatment at the concentration of 100 mg L\(^{-1}\) or lower had a promotive effect on the wheat haploid production.

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