Euphytica **49**: 161–168, 1990.

Simplified culture method of detached ears and its application to vernalization in wheat

K. Kato, S. Tomo, S. Yamazaki & K. Hayashi
Faculty of Agriculture, Kochi University, Nankoku, 783, Japan

Received 26 October 1989; accepted in revised form 20 February 1990

Key words: Triticum aestivum, bread wheat, detached ear culture, immature seed vernalization, narrow-sense earliness, photoperiodic response

Summary

The effects of the addition of sulfurous acid into culture solution and of cold treatment of the solution were examined to simplify the culture of detached wheat ears. In the simplified method, detached ears could be cultured at room temperature on the liquid medium containing 100 g/l sucrose and 0.075% sulfurous acid without any sterilization. The immature seeds in detached ears cultured by this method were treated with low temperature or with chemicals known to have vernalizing effect. The chemical treatment did not affect the chilling requirement of immature embryos, although photoperiodic response and narrow-sense earliness were reduced by kinetin and trypsin. The low temperature treatment drastically affected the chilling requirement, and fully vernalized mature seeds having normal germinability were obtained by treating the detached ears in culture with low temperature from 10 days after anthesis.

Introduction

Heading acceleration through artificial treatment is desirable for wheat breeding and genetical research. However, in some varieties, chilling treatment for more than two months is necessary for vernalization.

Chemical treatment of sprouting seeds (Barabas & Csepely, 1978), and low temperature and chemical treatment of intact immature seeds (Weibel, 1958; Pauli et al., 1962) were examined as the alternative vernalization methods. However, there is no chemical which is effective enough to change winter growth habit into spring growth habit. As to the vernalization of intact immature seeds, there is a difficulty in synchronization of flowering among tillers in spite that it is important to get uniformly treated seed in large amount. On the other hand, the use of detached ears (Pauli et al., 1962; Suge, 1970) makes it possible to treat all tillers at the same stage, though the treatment date may be different among tillers. However, it was difficult to get well matured seeds, because detached ears were supplied no nutrition. A new method to vernalize immature embryos can be established, if it becomes possible to supply nutrition to immature caryopses in detached ear.

After Jenner (1968) reported that starch content of immature caryopses increased through the culture of detached ears on sucrose solution, Donovan & Lee (1977) and Singh & Jenner (1983) improved this culture method and succeeded in getting well matured seeds by aseptic culture. Kato & Hayashi (1985) tried to simplify this method for chemical or physical treatment of immature embryos. Germinable seeds could easily be obtained by the culture of detached ears on sucrose solution without sterilization. However, there remained several prob-

lems, such as small grain size and often decay of culture solution and stem base.

The effects of the addition of sulfurous acid into culture solution (Yasuda, 1948) and of cold treatment of the solution (Donovan & Lee, 1977) were examined in the present study to overcome the above problems, resulting in the establishment of a simplified and reliable culture method of detached ears. Using this method, immature seeds were treated with low temperature or with chemical substances, and the treatment effects on heading were evaluated.

Materials and methods

Establishment of culture method

A highly spring wheat variety 'IL 171' was grown in the field in 1985/86. When terminal spikelet just emerged from the leaf sheath, the ear was cut with stem at the middle of the second internode and leaf blades were removed. The basal part of such detached ear was put into a test tube containing culture solution without plug. The detail of culture conditions in each experiment is described below. Grains became dry and hard after about one month. Fertility measured as grain number per spikelet, grain weight per ear and average one grain weight were used as the criteria to determine the best culture condition.

Experiment 1. Sulfurous acid and cold treatment. Detached ears were cultured on 100 g/l sucrose solution (Kato & Hayashi, 1985) containing four concentrations (0, 0.075, 0.15 and 0.225%) of sulfurous acid, combined with or without cold treatment of the solution. Cold treatment was carried out by dipping the lower part of test tube in water bath kept at 5° C. The controls were maintained at room temperature (ca. 25° C). Nine ears were cultured in each treatment. Besides the characters mentioned above, volume of culture solution decreased in 24 hours was measured at an interval of 10 days from the beginning of culture. Although this measure was the sum of absorption by detached ears and evaporation, the quantity of evaporation was small enough to be neglected.

Experiment 2. Sucrose concentration. The optimum concentration of sulfurous acid was proved to be 0.075% from the result of experiment 1. Therefore, detached ears were cultured on 0.075% sulfurous acid solution containing seven concentrations (0, 25, 50, 75, 100, 150 and 200 g/l) of sucrose, combined with or without cold treatment of the solution. Nine ears were cultured in each treatment.

Treatment of immature seeds in detached ears

Three wheat varieties covering a wide range of chilling requirement, that is, a winter wheat 'Fukoku', a spring wheat 'IL 13' and a highly spring wheat 'Norin 42', were grown in the field in 1985/86. Just-headed ears were detached and cultured in the same way as mentioned before. For the cold treatment of immature seeds, the detached ears were first cultured by the standard method, that is, on the solution containing 100 g/l sucrose and 0.075% sulfurous acid, at room temperature with cold treatment of the solution. Five, ten or fifteen days after anthesis, they were transferred into an incubator kept at 5° C. After 60 days at 5° C, they were again maintained at room temperature until their maturation. For chemical treatment, detached ears were first cultured by the standard method until 10 days after anthesis. They were then cultured by the standard method until their maturation, except that the culture solution contained the chemicals whose vernalizing effect had been reported. The chemicals and the concentrations examined in the present study were 1×10^{-6}M kinetin (Barabas & Csepely, 1978), 1×10^{-4}M gibberellic acid (Weibel, 1960), 1×10^{-3}% trypsin (Tomita, 1973), 1×10^{-4}M cytidylic acid (Tomita, 1968) and 1×10^{-4}M uracil (Suge & Yamada, 1964). Besides these, detached ears were also cultured by the standard method as a control to check treatment effect. Fifteen ears were cultured in each treatment.

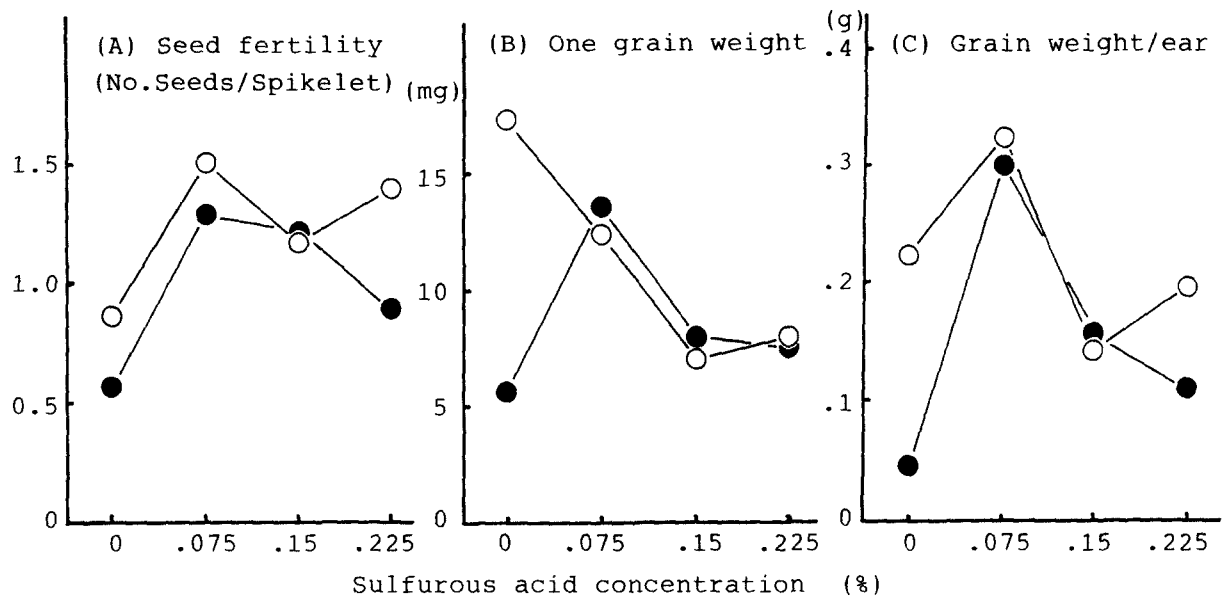

Fig. 1. Effects of sulfurous acid concentration and of cold treatment on the culture response of detached ears. (○, Cooled; ●, Not cooled).

Three heading traits, chilling requirement, photoperiodic response and narrow-sense earliness of treated and control plants were evaluated. For the evaluation of chilling requirement, just sprouted seeds were subjected to chilling treatment at 5° C for various periods from 0 to 70 days, and then grown in phytotron under a 20° C and 24 h day-length regime. Chilling requirement, which was expressed as the minimum duration of chilling treatment necessary for full vernalization, was evaluated by comparing the days from the first leaf unfolding to flag leaf unfolding (D1f) among treatment durations. Twelve plants were examined in each case. The detail of the cultivation and calculation methods is given in Kato & Yamagata (1988). For the evaluation of photoperiodic response and narrow-sense earliness, just sprouted seeds were fully vernalized by chilling treatment at 5° C for 70 days and then grown in phytotron kept at 20° C under a 12 h or 24 h day-length regime. The days from the end of chilling treatment to flag leaf unfolding under a 24 h day-length regime and its difference between day-length regimes were regarded as narrow-sense earliness and photoperiodic response, respectively (Takahashi & Yasuda, 1958). Six plants were examined in each treatment.

Results

Establishment of culture method

Experiment 1. Sulfurous acid and cold treatment. Although seed fertility tended to be improved by sulfurous acid (Fig. 1A), it was statistically insignificant. One grain weight increased at 0.075% sulfurous acid concentration ($P < 0.01$) (Fig. 1B). Grain weight per ear was the highest at 0.075% and decreased with the increase of sulfurous acid concentration ($P < 0.01$). The effect of cold treatment on grain weight per ear was positive in the absence of sulfurous acid ($P < 0.01$). However, it was statistically insignificant in the presence of sulfurous acid (Fig. 1C).

The amount of culture solution absorbed by the detached ears generally decreased in the course of culture in each treatment. As the decrease was the least, from 4.44 ml on the 1st day to 2.0 ml on the 20th day, at 0.075% sulfurous acid concentration,

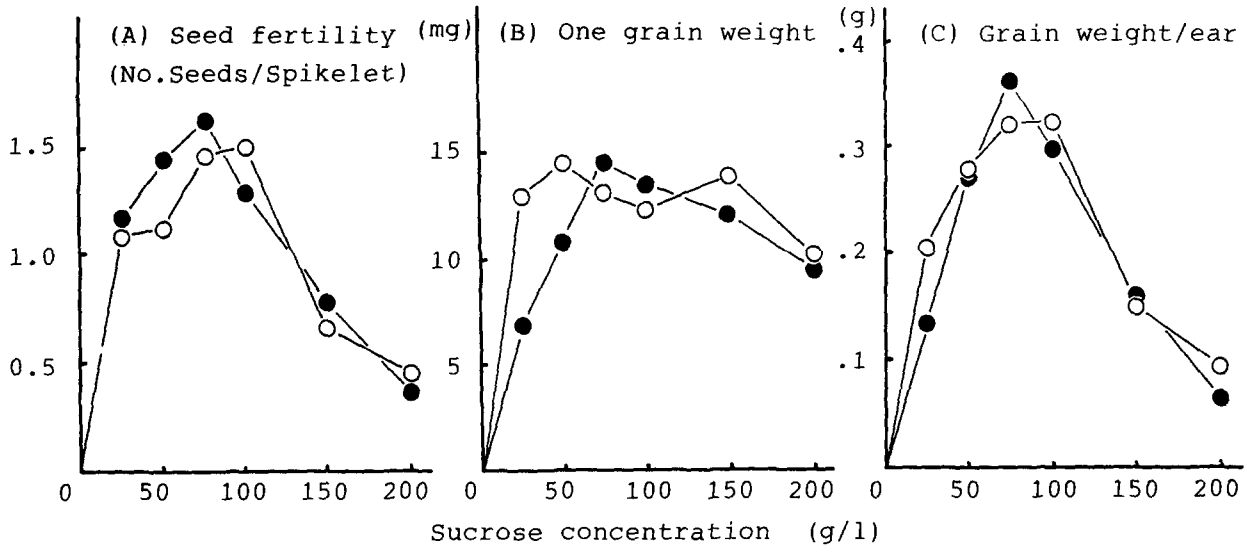

Fig. 2. Effects of sucrose concentration and of cold treatment on the culture response of detached ears. (○, Cooled; ●, Not cooled).

more than double solution compared to sulfurous acid-free solution was absorbed on the 10th day and the 20th day from the beginning of culture. Such advantage in absorption capacity is considered to explain the increase in one grain weight and grain weight per ear. On the other hand, cold treatment did not affect the absorption capacity of detached ears.

It was concluded from these results that the addition of 0.075% sulfurous acid into the solution was more effective than cold treatment of the solution to improve the culture method through the sup-

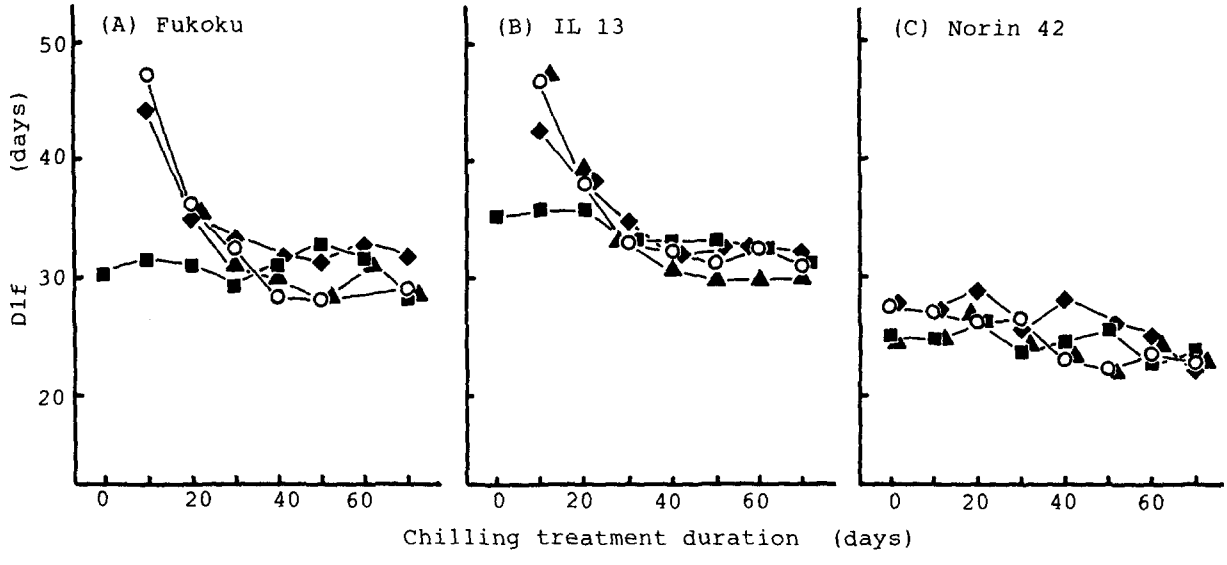

Fig. 3. Effect of chilling treatment on D1f of the plants raised from the seeds treated with low temperature or kinetin. (○, Control; ■, ◆, Treated with low temperature from 10 days (■) and 15 days (◆) after anthesis; ▲, Treated with kinetin).

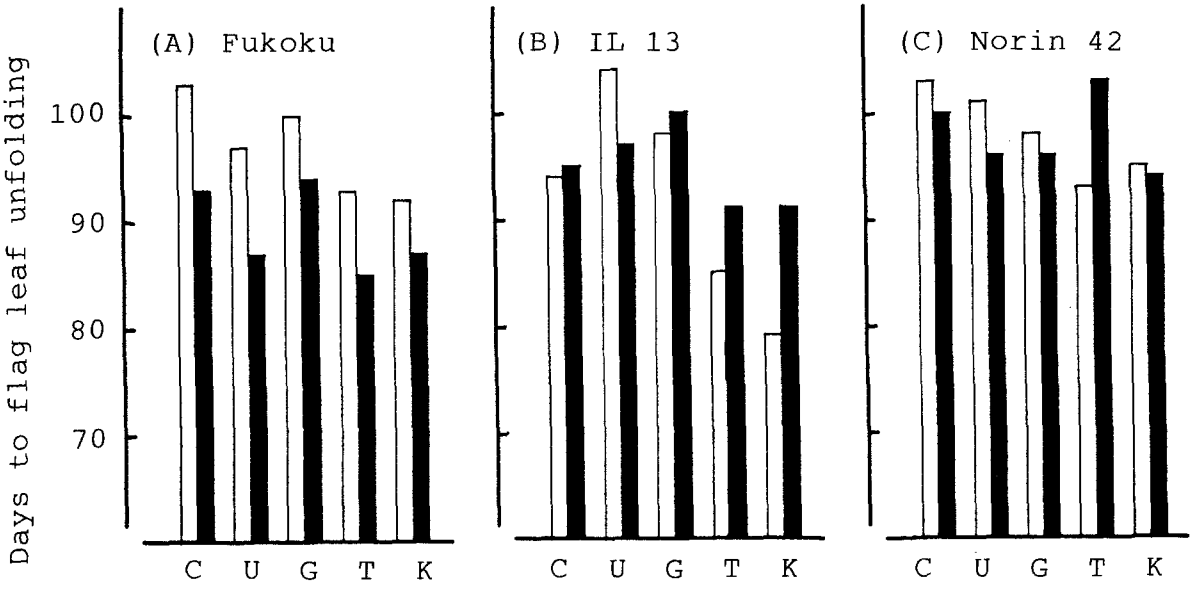

Fig. 4. Effect of chemical treatment on the days to flag leaf unfolding of fully vernalized plants. Plants were grown under 12 h (□) or 24 h (■) day-length regime. Days to flag leaf unfolding is expressed in the relative values of control plants. (C, Cytidylic acid; U, Uracil; G, Gibberellic acid; T, Trypsin; K, Kinetin).

pression of often decay of culture solution and stem base.

Experiment 2. Sucrose concentration. As shown in Fig. 2, fertility, one grain weight as well as grain weight per ear increased with the increase of sucrose concentration from 0 to 50 or 100 g/l. However, grain weight per ear drastically decreased with the increase of sucrose concentration from 100 to 200 g/l, mainly due to the reduction in seed fertility. Such a tendency was independent of the cold treatment, except that one grain weight increased with cold treatment at a 25 g/l sucrose concentration. Therefore, it was concluded that the optimum sucrose concentration was from 50 to 100 g/l, when 0.075% sulfurous acid was added into the solution or the solution was cooled.

Treatment of immature seeds in detached ears

Mature seeds with normal germinability were obtained, even when immature seeds were treated with low temperature or chemicals during grain filling period. However, by low temperature treatment from 5 days after anthesis, one grain weight, grain weight per ear and seed germinability decreased exceptionally, though seed fertility was hardly influenced. Therefore, the effect of low temperature treatment from 5 days after anthesis on heading traits could not be evaluated.

The change in D1f with the increase of the duration of chilling treatment given after sprouting is shown in Fig. 3 for the evaluation of chilling requirement. In winter wheat variety 'Fukoku', D1f of control plants decreased with the increase of chilling treatment duration, and then became constant by the treatment for more than 40 days.

Therefore chilling requirement of 'Fukoku' was evaluated at 40 days. As to the plants raised from the seeds treated with low temperature from 10 days after anthesis, the change in D1f by chilling treatment was statistically insignificant, indicating that 'Fukoku' had been fully vernalized by this pre-treatment. On the other hand, the acceleration of flag leaf unfolding by the treatment from 15 days after anthesis was not so large, and D1f was decreased by chilling treatment. Therefore it was sug-

gested that 'Fukoku' had been partially vernalized by this pre-treatment. Chilling requirement of spring wheat variety 'IL 13' was evaluated at 30 days (Fig.3B). And 'IL 13' was also fully vernalized by the treatment from 10 days after anthesis and partially from 15 days after anthesis. On the other hand, in highly spring wheat variety 'Noris 42', D1f of control plants showed constant value irrespective of chilling treatment duration, indicating that chilling requirement was 0 days (Fig. 3C). Therefore, further reduction in chilling requirement did not occur even by the pre-treatment with low temperature.

The D1f of the plants treated with kinetin changed with the increase of chilling treatment duration in the same way as that of the control plants irrespective of the variety, indicating that the pre-treatment with kinetin had no effect on chilling requirement (Fig. 3). The same tendency was also observed in the plants treated with the other chemicals (Data was not shown).

Fig. 4 shows the days to flag leaf unfolding of plants raised from chemically treated seeds expressed in relative values of control plants. Flag leaf unfolding under a 24 h day-length regime was accelerated by kinetin, uracil and trypsin in 'Fukoku' ($P < 0.01$), by kinetin and trypsin in 'IL 13' ($P < 0.05$) and by kinetin in 'Norin 42' ($P < 0.05$). As the plants were fully vernalized by chilling treatment given after sprouting, these results indicated that narrow-sense earliness was shortened by the treatment with these chemicals. The days to flag leaf unfolding under a 12 h day-length regime, which was controlled by photoperiodic response and narrow-sense earliness, tended to be shortened by kinetin and trypsin in three varieties. In variety 'IL 13', whose photoperiodic response was rather strong, the acceleration of flag leaf unfolding by such treatment was statistically significant ($P < 0.01$). The difference in the days to flag leaf unfolding between 12 h and 24 h day-length regimes, which showed photoperiodic response itself, was also reduced in 'IL 13', though it was not reduced in less sensitive varieties 'Fukoku' and 'Norin 42' (Table 1). These results showed that photoperiodic response and narrow-sense earliness were reduced by both chemicals. It was concluded that the chemicals known to have vernalizing effect, were not effective in reducing chilling requirement, and that some chemicals, such as kinetin and trypsin, could accelerate heading by reducing narrow-sense earliness and photoperiodic response.

Table 1. Effect of chemical treatment on photoperiodic response

Chemical	Variety and photoperiodic response (days)		
	Fukoku	IL 13	Norin 42
Control	6.3	14.5	4.4
Cytidylic acid	9.7	13.5	5.2
Uracil	9.2	17.0	5.4
Gibberellic acid	8.0	13.6	4.9
Trypsin	8.2	10.7	2.0
Kinetin	7.3	8.3	4.3

Discussion

Establishment of culture method

The vernalizing effect of chemical substances can be studied in culture, if immature wheat embryos treated with chemicals can develop and mature normally. Kato & Hayashi (1985) tried to simplify the culture method of detached ears for this purpose, and obatined germinable seeds even without any sterilization. Although this method was applicable to more than 100 wheat varieties, it was still necessary to change culture solution and to cut off stem base every 2 or 3 days because of often decay of the solution and stem base. In the present study, the addition of sulfurous acid into culture solution was found to be effective in suppressing such decay. The addition of 0.075% sulfurous acid enhanced the absorption of culture solution, resulting in the increase in grain weight per ear through the increase in sucrose uptake. Although similar effect was also observed by cold treatment of culture solution, chilling was less effective for the increase in absorption capacity and grain weight per ear. According to Yasuda (1948), the addition of sulfu-

rous acid enhanced the absorption of water in detached spikes of sugarcane.

The pH of culture solution changed from 5.8 to 2.4 by the addition of 0.075% sulfurous acid. However, detached ears cultured on the solution whose pH was adjusted at 2.4 by HCl had lower grain weight per ear than those cultured on sulfurous acid-free solution (Kato & Hayashi, unpublished). Apparently, the positive effect of sulfurous acid was not due to change in pH of culture solution.

The optimum sucrose concentration of culture solution was so far reported to be from 20 g/l to 50 g/l (Jenner, 1968,1970; Donovan & Lee,1977; Singh & Jenner,1983). However, in the present study, it was rather high and ranged from 50 g/l to 100 g/l. Though the reason for such discrepancy is not known, it may be ascribable to the difference in the variety and in the culture method.

Kato & Hayashi(1985) reported that the optimum culture temperature was at 15° C, as both one grain weight and grain weight per ear decreased at higher temperature. However, grain filling period was about two months at 15° C and was twice longer than at 20° C and 25° C. It was suggested from these results that there were two kinds of optimum temperatures: 15° C to get large grain, and from 20° C to 25° C to save the time without any loss in germinability. Therefore detached ears were cultured at room temperature (ca. 25° C) in the present study.

Treatment of immature seeds in cultured ears

Using the culture method established above, it was shown that low temperature treatment was and the chemicals examined were not effective for the vernalization of immature embryos, though some chemicals were effective for narrow-sense earliness and photoperiodic response.

Embryo stage is an important factor for vernalization of immature seeds. Chilling treatment had to be started from 9 to 12 days after anthesis in wheat (Weibel, 1958) and by 14 days after anthesis in rye (Gregory & Purvis, 1938). In the present study, both winter and spring wheat cultivars were vernalized fully by the treatment with low temperature from 10 days after anthesis, but partially by the treatment from 15 days after anthesis.

The vernalizing effect of chemicals may not be ruled out altogether by the present results, because the effective concentration may be different depending on the treatment method, and we used similar concentrations as have been employed previously in other methods. On the other hand, some reports showed negative results on the vernalizing effect of chemical substances, such as kinetin (Tomita,1964; Sharma & Gill,1982) and gibberellic acid (Gott,1961). Furthermore, even in the reports that showed positive results of chemical vernalization:

1. The chemicals were used at low temperature, except Pauli et al.(1962) and Tomita(1964, 1973), therefore, heading acceleration might be due to chilling treatment or the interaction of two treatments,
2. Vernalizing effect was evaluated by the days from the end of chilling treatment to flag leaf unfolding or to heading, resulting in the confusion with the effect on narrow-sense earliness (Kato & Yamagata,1988). Tomita (1973) grew wheat plants under a natural day-length regime, resulting in the confusion even with the effect on photoperiodic response, and
3. No report so far showed full vernalization of winter wheat by chemical treatment alone.

Further studies may be necessary to determine the possibility of chemical vernalization.

In conclusion, the easiest way to get a higher number of germinable seeds from detached wheat ears is to culture the ear at room temperature on the liquid medium containing 100 g/l sucrose and 0.075% sulfurous acid without any sterilization. Although the grains were smaller in size than those obtained in aseptic culture reported by Singh & Jenner (1983), the present method should be suitable for chemical or physical treatment of immature embryos because of its easiness, being applicalbe to physiological study on embryo development and rescue of the interspecific hybrid embryos. It was experimentally confirmed that the present method was applicable also to vernalization treatment. Fully vernalized dry seed obtained by culturing at low temperature possesses the same

genetic constitution as the original seed, but is different in chilling requirement. Such material is equivalent to the seed of iso-genic line carrying *Vrn 1*. Therefore the present method should contribute to the genetical and physiological research on heading traits. Furthermore generation acceleration method should become more efficient by the use of this vernalization method, because chilling treatment can be accomplished during grain filling period.

References

Barabas, Z. & I.T. Csepely, 1978. Shortening vernalization of winter wheat with kinetin. Euphytica 27: 831–835.

Donovan, G.R. & J.W. Lee, 1977. The growth of detached wheat heads in liquid culture. Plant Sci. Letters 9: 107–113.

Gott, M.B., 1961. Flowering of Australian wheats and its relation to frost injury. Aust. J. Agric. Res. 12: 547–565.

Gregory, F.G. & O.N. Purvis, 1938. Studies in vernalization of cereals. II. The vernalization of excised mature embryos, and of developing ears. Ann. Bot. 5: 237–251.

Jenner, C.F., 1968. Synthesis of starch in detached ears of wheat. Aust. J. Biol. Sci. 21: 597–608.

Jenner, C.F., 1970. Relationship between levels of soluble carbohydrate and starch synthesis in detached ears of wheat. Aust. J. Biol. Sci. 23: 991–1003.

Kato, K. & K. Hayashi, 1985. Modification method to obtain mature dry seeds by succrose solution culture of detached wheat ears. Res. Rep. Kochi Univ. 33 (Agric. Sci.): 63–70 (in Japanese).

Kato, K. & H. Yamagata, 1988. Evaluation of chilling requirement and narrow-sense earliness of wheat cultivars. Jap. J. Breed. 38: 172–186.

Pauli, A.W., J.A. Wilson & F.C. Stickler, 1962. Flowering response in winter wheat as influenced by temperature, light, and gibberellic acid. Crop Sci. 2: 271–274.

Sharma, H.C. & B.S. Gill, 1982. Effect of embryo age and culture media on plant growth and vernalization response in winter wheat. Euphytica 31: 629–634.

Singh, B.K. & C.F. Jenner, 1983. Culture of detached ears of wheat in liquid culture: Modification and extension of the method. Aust. J. Plant Physiol. 10: 227–236.

Suge, H. & N. Yamada, 1964. Chemical control of plant growth and development. (4). Promotion of flowering induced by uracil, uridylic acid and several growth regulators in winter wheat. Proc. Crop Sci. Soc. Japan 32: 77–80.

Suge, H., 1970. Physiological aspects of flowering in winter wheat and barley. Bull. Natl. Inst. Agric. Sci., Ser. D. 17: 21–73 (in Japanese).

Takahashi, R. & S. Yasuda, 1958. Genetic studies on heading time in barley (in Japanese). In: K. Sakai, R. Takahashi & H. Akemine (Eds), Studies on bulk method of plant breeding, pp. 44–64. Yokendo, Tokyo.

Tomita, T., 1964. Studies of vernalization and flowering substances. IV. Bioassay of flowering activities of natural and chemical substances by the injection into the endospermic space of young winter wheat seedlings. Tohoku J. Agr. Res. 15: 1–7.

Tomita, T., 1968. Flowering activities of natural and chemical substances injected into the cavity of empty endospermic space of young winter wheat seedlings. In: F. & G. Wightman Setterfield (Eds), Biochemistry and psysiology of plant growth substances. Runge Press, Ottawa, pp. 1399–1413.

Tomita, T., 1973. Crop diagnostic study and its application: Vernalization in winter wheat caused by protease. Bull. Natl. Inst. Agric. Sci., Ser. A. 20: 1–16.

Weibel, D.E., 1958. Vernalization of immature winter wheat embryos. Agron. J. 50: 267–270.

Weibel, R.O., 1960. Effect of gibberellin on the vernalization period of winter wheat. Agron. J. 52: 122–123.

Yasuda, S., 1948. Reproduction physiology in higher plants. Yokendo, Tokyo. (in Japanese).