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FACTORS INFLUENCING SEEDLING EMERGENCE FROM IMMATURE EMBRYOS OF WINTER WHEAT AND THE TRANSPLANTATION IN SUMMER FIELD

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Abstract

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In order to complete 4-5 generations development of winter wheat per year via taking timely use of summer field and greenhouse, the study was performed to investigate the influencing factors on the seedlings mergence from immature embryos of winter wheat and their transplanting survival rate in summer field. The immature embryos of Jimai 20, Yanyou 361, Tainong 18, Zhongyou 9507 and F_1 (Jimai20×Yanyou361) at 11-13, 14-16 and 17-19d of embryonic age were cultured in three different media with or without hormones. The seedling emergence rates were compared and the seedlings were directly transplanted to summer field after vernalization for 15, 20 and 25d, respectively. The results showed that the appropriate time for vernalization of seedlings was 20-25d; Among the nine inoculation tests, higher germination rate (95.80% on average) and transplanting survival rate of seedling (86.95% on average) were obtained from the 14-16d immature embryos cultured in the media without hormones, and the seedling emergence rate was the highest (82.95% on average). When seedlings were transplanted to the groove in summer field, we found the transplanting survival rate was greatly improved by covering the roots of seedlings with a small amount of detritus substrates and burying in soil. In summary, we have identified and optimized critical factors that influence the seedling emergence from immature embryos of winter wheat and their transplanting survival rate in summer field.

Key words: immature embryo, in vitro wheat culture, seedling emergence rate, vernalization, transplantation

Inroduction

Wheat is the staple food for 40% of the world's population (Gupta et al., 2008), so shortening the breeding cycle becomes an important topic for wheat breeding. Breeding experts usually carry out adding generation breeding by making use of the climates of different regions with bigger difference in latitude or altitude and so achieves good breeding effect (Gupta et al., 2008). Wu et al. (1998) used a common technology for adding generation of wheat, which needs mature seeds of wheat with stronger springness, but has a greater limitation and requires longer growth cycle. Since Shimada (1978) successfully acquired regenerated plants of wheat by immature embryo culture, immature embryo has been considered as one of the most effective and ideal explants sources for wheat tissue culture (Agarwal and Tiwari 1995). Immature embryo culture has been widely used in genetic breeding of wheat (Vasil et al., 1992, 1993;Becker et al., 1994; Xia et al., 1999). Ding et al. (2005) transplanted immature embryo plants to summer greenhouse for generation adding by using the immature embryos culture in vitro. Since this method requires blowing to the greenhouse to reduce temperature, its cost is very high. At present, the research on fast breeding techniques by directly culturing wheat immature embryos into seedlings are rarely seen, and the systematic research on appropriate embryonic age of immature embryos, medium, vernalization time and transplanting survival of immature embryos in summer field to directly develop into seedlings has not been reported yet. For culture of immature embryos of winter wheat to grow into seedlings, the key of the technol-

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ogy system is to improve the germination rate and transplanting survival rate of immature embryos plants in the summer field. For this reason, we studied the embryonic age, culture medium, germination rate of immature embryos, vernalization time and transplanting survival rate of immature embryo seedlings, and determined the appropriate conditions for inoculating immature embryos of the three embryonic age 11-13, 14-16 and 17-19d to culture media MB, A and B respectively. The purpose of this study is to shorten the breeding cycle with immature embryo culture of winter wheat and to provide reference for the full use of natural light and temperature in summer to speed up the breeding process.

Materials and Methods

This experiment was processed at agricultural experimental field and screened insectproof house of Shandong Agricultural University, and Tai'an laboratory of National Wheat Improvement Center. The test materials Jimai 20, Yanyou 361, Tainong 18 and Zhongyou9507 were preserved by Tai'an laboratory of National Wheat Improvement Center, and the F1 between Jimai 20 and Yanyou 361 was created by the research group.

Three types of medium were used in this experiment. MB culture media: MS macroelement + B5 microelement + sucrose 30g/L + agar 8g/L + casein hydrolysate 0.5g/L; Culture media A: MB culture media + 1-2-chloro-4-pyriddyl-3-phenycurea 0.17 mg/L + 3-indole bueyric acid 0.5 mg/L; Culture media B: MB culture media + 6-benzylaminopurine 0.05 mg/L + 2-naphehyl acetic acid 0.13 mg/L.

The soil and fertilizer condition was organic soil manure 100 m^3/mu and NPK complex fertilizer 20 kg/mg. The field was north-high and south-low terrain which was favorable for drainage.

For three consecutive years (October 6, 2008, October 7, 2009 and October 5, 2010), Jimai 20, Yanyou 361, Tainong 18 and Zhongyou 9507 were planted every year in the experimental plots of Shandong Agricultural University.

The pollination time of single spike was labeled during wheat flowering time within the first ten days of May each year, followed by creating F_1 between Jimai 20 and Yanyou 361 using the conventional cross hybridization.

The 11-13d, 14-16d and 17-19d immature embryos of the 5 kinds of materials were inoculated to MB, medium A and medium B, respectively, and nine inoculation experiments were designed. In the middle and late of May each year, 600 immature embryos with different embryonic age were taken from each tested wheat species, and disinfected with 75% ethanol. The immature embryos were washed thrice with sterile water and then extruded. Scutum was placed downward which was favorable to direct germination of immature embryos according to previous reports (Sears and Deckard, 1982; Maddock et al., 1983; Ozias-Akins and Vasil, 1982), 200 immature embryos were inoculated to MB, medium A and medium B**,** respectively, and were cultured at 25^0C in light incubator.

The immature embryos with buds were transferred to 0-6^0C light incubator, and at this low temperature, the vernalization of seedlings received three treatments, 15d, 20d and 25d.

The seedlings with different vernalization treatments were transferred to 25^0C + light incubator for 2d hardening and then outdoor for 1d hardening. In June of each summer, the seedlings were transplanted to the screen-house of Shandong Agricultural University with adequate basal fertilizer and watering. Then the seedlings were transplanted to the groove in north-to-south direction. The roots of seedlings were covered with a small amount of detritus substrates and then with soil. Within one week after seedling transplantation, shading net was used and at 10d after seedling transplantation, nitrogen topdressing 10kg/mu was applied to promote plant growth.

The calculation formulas used in this experiment were as follows. Germination rate (%) = the amount of seedlings/total number of inoculated immature embryos × 100%; transplanting survival rate of seedlings (%) = the number of seedlings survived after transplanting /the total number of seedlings transplanted; the seedling rate (%) = the germination rate × the transplanting survival rate of seedlings. DPS software was used to analyze the significance of the result of the experiments.

Results

Seedlings from 14-16d immature embryos culturing 3d on the B medium. Seedlings before transplanting and tansplant survival Seedlings are separately showed in part Figure 1 A, B and C.

The germination rate and the transplanting survival rate of seedlings at different embryonic ages after culture in the same medium were different. The immature embryos of three embryonic ages were inoculated to MB medium, and the germination rate and transplanting survival rate are shown in Figure 2. The germination rate of 11-13d embryonic age of 5 types of test materials ranged from 67.00% to 71.00%, with an average of 69.10%; the transplanting survival rate of 11-13d embryonic age was 13.14%- 21.83%, with an average of 17.03%; the germination rate of 14-16d embryonic age was 94.50% - 97.00%, with an average of 95.80%; the transplanting survival rate of 14-16d embryonic age was above 80.00%, with an average of 86.95%; the germination rate of

17-19d embryonic age was 14.50% -19.00%, with an average of 16.20%; the transplanting survival rate of 17-19d embryonic age ranged from 90.32% to 94.78%, with an average of 92.53%. Therefore, with regard to the immature embryos in MB medium, the germination rate of 14-16d embryonic age was the highest (95.80% on average), and that of 17-19d embryonic age was the lowest (16.20% on average); the transplanting survival rate of 17-19d embryonic age growing into seedlings was the highest (92.53% on average), while that of 11-13d embryonic age was the lowest (17.03% on average);

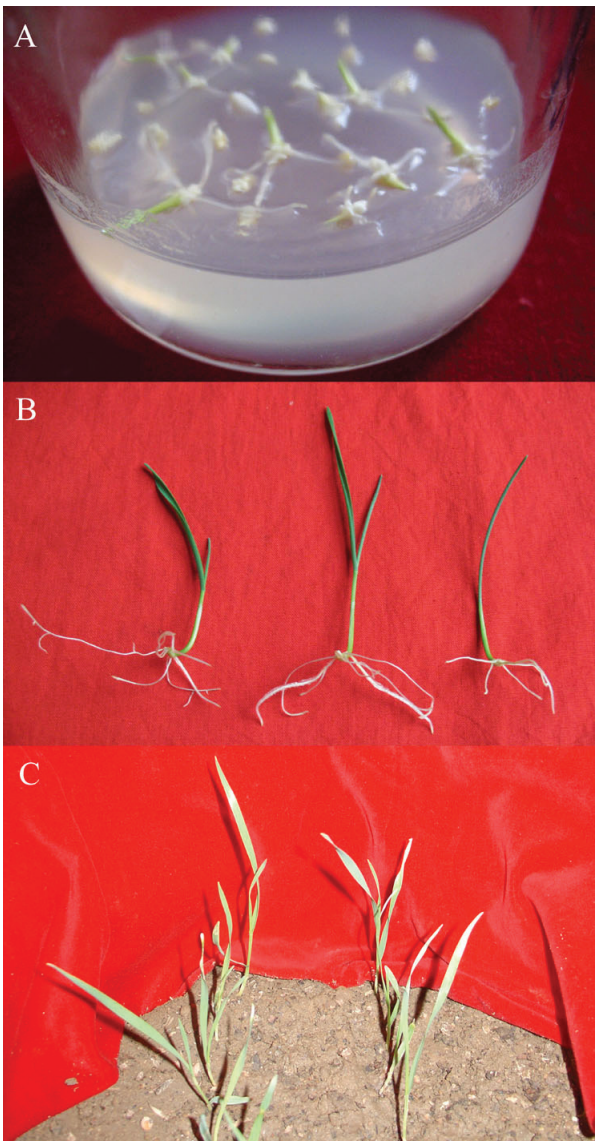

Fig. 1. Seedlings from 14-16d immature embryos cultured 3d on the B medium, before transplanting and transplant survival seedlings are showed in A, B and C separately

the germination rate of immature embryos of the same embryo age but different genotype materials varied slightly, and the difference in the transplanting survival rate of seedlings was not obvious. This finding is similar to the results of Ye et al. (1998), but different in germination rate of immature embryos of different embryonic ages and in transplanting survival rate are greater.

The immature embryos of three embryonic ages were inoculated to medium A, and the germination rates and transplanting survival rates are shown in Figure 3. The results showed that on medium A, the germination rate of 11-13d immature embryos was the highest, with the average value of 98.30% among the five test materials; the germination rate of 17-19d immature embryos was the lowest, with an average of 76.90%; the transplanting survival rate of 17-19d embryonic age was the highest, with an average of 68.36%, while that of 11-13d embryonic age was the lowest with an average of 9.55%; the germination rate of immature embryos of the same embryonic age but the materials with different genotypes varied slightly, and the difference in the transplanting survival rate of seedlings was not obvious, but the differences in germination rate and transplanting survival rate of different embryonic ages were great. This is the same as the conclusion obtained on MB medium.

The immature embryos of three embryonic ages were inoculated to medium B, and the germination rates and transplanting survival rates are shown in Figure 4. The results showed that on medium B, the germination rate of 17-19d immature embryos was the highest, with an average of 95.20% among the five test materials; the germination rate of 11-13d immature embryos was the lowest, with an average of 33.99%; the transplanting survival rate of 17-19d immature embryos was the highest, with an average of 49.92%, while that of 11-13d immature embryos was the lowest with an average of 7.53%; the germination rate of the same embryonic age but the materials with different genotypes varied slightly, and the difference in the transplanting survival rate of seedlings was not obvious. But the differences in germination rate and transplanting survival rate of different embryonic ages were great. This is the same as the conclusion obtained on MB medium or medium A.

We compared the germination rate and transplanting survival rate (Figures 2, 3 and 4) of the nine treatments when the immature embryos of three embryonic ages were inoculated to three kinds of media. The results showed that the germination rate of 11-13d immature embryos inoculated to medium A was the highest, and the average among the five kinds of test materials reached 98.30%; the transplanting survival rate of 17-19d embryonic age inoculated to MB medium was the highest, and the average among the five kinds of test mate-

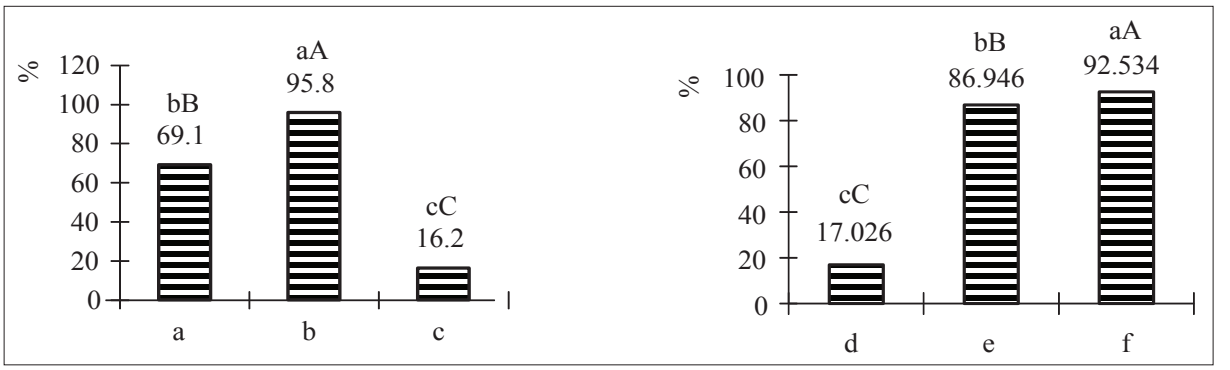

Fig. 2. The germination rate of immature embryo (left) and transplant survival rate (right) of seedlings on MB medium
Note: a -The average Germination rate of 11-13d immature embryo; b -The average Germination rate of 14-16d immature embryo; c -The average Germination rate of 17-19d immature embryo; d -The average Transplant survival rate of seedlings from 11-13d immature embryo; e- The average Transplant survival rate of seedlings from 14-16d immature embryo; f- The average Transplant survival rate of seedlings from 17-19d immature embryo

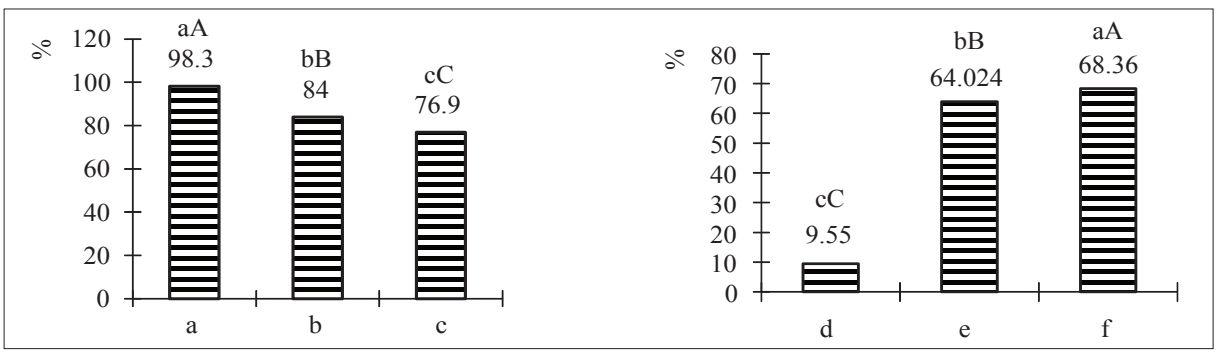

Fig. 3. The germination rate of immature embryo (left) and transplant survival rate (right) of seedlings on A medium
Note: a- The average Germination rate of 11-13d immature embryo; b -The average Germination rate of 14-16d immature embryo; c- The average Germination rate of 17-19d immature embryo; d- The average Transplant survival rate of seedlings from 11-13d immature embryo; e -The average Transplant survival rate of seedlings from 14-16d immature embryo; f -The average Transplant survival rate of seedlings from 17-19d immature embryo

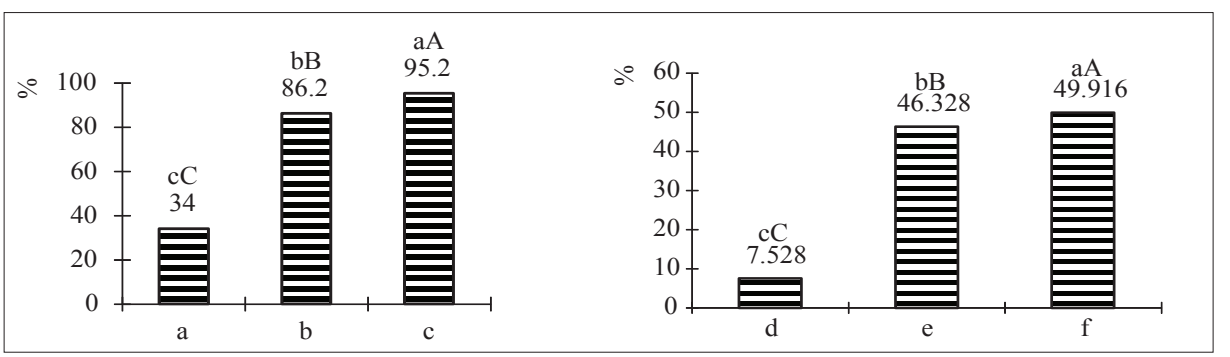

Fig. 4. The germination rate of immature embryo (left) and transplant survival rate (right) of seedlings on B medium
Note: a -The average Germination rate of 11-13d immature embryo; b -The average Germination rate of 14-16d immature embryo; c- The average Germination rate of 17-19d immature embryo; d -The average Transplant survival rate of seedlings from 11-13d immature embryo; e -The average Transplant survival rate of seedlings from 14-16d immature embryo; f -The average Transplant survival rate of seedlings from 17-19d immature embryo

rials reached 92.53%. Among the nine treatments, the immature embryos with high germination rate might have high transplanting survival rate, while that with low germination rate might have low transplanting survival rate. There was no treatment in which the highest germination rate coexisted with highest transplanting survival rate or the lowest germination rate with the lowest transplanting survival rate. The growth of immature embryos of winter wheat into seedlings requires not only high germination rate but also seedlings' high transplanting survival rate. In order to better evaluate and select the optimal combination of the two factors, embryonic age and medium, the germination rate was multiplied by transplanting survival rate as the seedling rate. The higher the seedling rate, the more appropriate the medium for the culture of immature embryos. The comparison of the germination rates among the nine treatments (Table 1) showed that the germination rates of 11-13d immature embryos inoculated at medium MB, A and B were very low, with 11.77%, 9.39% and 2.56% respectively; the germination rate of 14-16d immature embryos inoculated at medium MB, A and B was 82.95%, 53.78% and 39.89%, respectively; the germination rate of 17-19d immature embryos inoculated to MB, media A, and B was 14.99%, 52.75%, and 47.52%, respectively. Therefore, it is the most appropriate for 14-16d immature embryos to be inoculated to MB medium to achieve a germination rate as high as 82.95%.

Three different vernalization treatment durations of seedlings were set as 15 d, 20d, and 25d, respectively. Flowering and fruiting were found in seedlings in all the three treatments. For seedlings after vernalization for 20 and 25 d, 35-40d was required from seedling transplanting to flowering, and the flowering stage of seedlings after vernalization for 20 and 25d showed no significant difference. Meanwhile, for seedlings after vernalization for 15d, 50-55d was required from seedling transplanting to flowering. However, no seedlings of low embryonic age were found to require shorter vernalization time.

The results above showed that the appropriate vernalization time for11-19d embryonic age was 20-25d.

The largest difficulty in seedling transplanting in summer field is to ensure the survival of seedlings. It is very hard for weak seedlings to survive when transplanted in summer, so we transplanted seedlings to the groove to protect the seedlings from being flooded. The roots of seedlings were covered with a small amount of detritus substrates and then with soil. Within one week after seedling transplantation, shading net was used. Thus the transplanting survival rate of seedlings in summer field was improved effectively. After 10 days of seedling transplantation, nitrogen topdressing at 10kg/mu was applied to promote seedling growth. The old seedling was decreased effectively and the seed setting rate was improved.

As shown in Table 1, the germination rate of 11-13d immature embryos in MB, medium A and medium B was 11.77%, 9.39% and 2.55%, respectively; the germination rate of 14-16d immature embryos in MB, medium A and medium B was 82.95%, 53.78% and 39.89%, respectively, which also decreased in sequence. This showed that it is appropriate to inoculate 11-16d immature embryos to MB medium containing no hormone; the germination rate of 17-19d immature embryos in the medium containing hormone increased obviously. The results above showed that 11-16d immature embryos are appropriate to be inoculated to MB medium containing hormone, while 17-19d immature embryos appropriate to be inoculated to media A and B containing hormone.

Discussion

There are controversies concerning the influence of embryonic age on seedlings, but it is most believed that the immature embryos of 1-1.5 mm diameter and 10-16d embryonic age are the most appropriate (Liang et al., 1988; Altpeter et al., 1996). Our results showed that the transplanting survival rate on the same kind of medium (Figures 2, 3 and 4) increased with embryonic age. The reason might be that the older the embryonic age and the greater the nutrient accumulated in embryo, the more mature the organ development and the stronger the ability of seedlings to absorb nutrition, so the seedlings will be better adapt to the environment and resist adverse stress. But for immature embryos of winter wheat to grow into seedlings, high germination rate is required. Comparison of the germination rates in the nine experiments (Table 1) showed that the 14-16d

Table 1
Seedling rate of three embryo ages immature embryo on three kinds of media, %

Medium	11-13 d Immature embryo	14-16 d Immature embryo	17-19 d Immature embryo
MB	11.77 fF	83.29 aA	14.99 eE
A	9.39 gF	53.78 bB	52.57 bB
B	2.56 hG	39.94 dD	47.52 cC

immature embryos (about 1 mm embryo diameter) were the most appropriate to be inoculated to MB medium, with the highest germination rate of 82.95%. Strongly influenced by environmental temperature, the optimal embryonic age in different regions tends to differ, which is consistent with the results by Liang and Gao (1986).

Since one-step seedling formation of wheat immature embryos is rarely studied, there are no consistent conclusions about what kind of medium has the optimal effect on the growth of wheat immature embryos into seedlings. The results of this study showed that the germination rate of 11-13d immature embryos in medium A was the highest as 98.30%, but the transplanting survival rate was as low as only 9.55%, indicating that the addition of 1-2-chloro-4-pyriddyl-3-phenycurea 0.17 mg/L and 3-indole bueyric acid 0.5 mg/L in the medium can promote germination of relatively young embryos (11-13d), but is unfavorable to transplanting survival of embryo seedlings, which may be due to the facts that relatively young embryos has less endogenous hormone and that exogenous hormones can accelerate the growth of seedlings but do not promise a strong plant. The germination rate of 14-16d immature embryos in MB, medium A and medium B was reduced in sequence, which is consistent with the results by Ding et al. (2005) and An et al. (2000). This indicates that the endogenous hormone of 14-16d immature embryos is enough for seedling emergence, and no additional hormones are needed. If exogenous hormones are added, the excess hormones might instead inhibit seedlings. The seeding roots that grew on MB medium were slender and long, and the plants were strong with fibrous roots, enabling easy transplanting survival. But the seeding roots that grew on medium A were thick and long, and the root was tender without fibrous root. The transplanting survival rate was low. The reason might be that after 1-2-chloro-4-pyriddyl-3-phenycurea was added, the medium containing auxin could obviously promote the formation of embryonic callus and embryogenesis (Carman et al., 1987). The roots of seedlings on medium B had poor ability to absorb soil nutrients because the roots were shorter without fibrous root. As a result, the transplanting survival rate was low. Meanwhile, it also showed that the medium could promote the growth of roots if 1-2-chloro-4-pyriddyl-3-phenycurea 0.17 mg/L and 3-indole bueyric acid 0.5 mg/L was added to the medium, but the addition of 6-benzylaminopurine 0.05 mg/L and 2-naphehyl acetic acid 0.13 mg/L inhibited the growth of the roots; The germination rate of larger immature embryos of 17-19d in the medium containing exogenous hormones increased significantly, but the transplanting survival rate reduced obviously. The reason might be that the endogenous hormones gradually reduced with the increase of the embryonic ages. Larger immature embryos of 17-19d needed to absorb exogenous hormones to accelerate seedling emergence. However, the exogenous hormones could accelerate the spindling but not make the root stronger. This led to the reduction in the transplanting survival rate of seedlings.

Flowering and fruition of winter wheat is not possible without appropriate vernalization treatment. This experiment revealed that it is appropriate to transfer immature embryos with roots and shoots into incubator at the temperature of 0 to 6^0C and 16 h light / 8 h dark for vernalization treatment for 20-25 d. The seedlings began flowering and fruition 35-40d after being transplanted. If the vernalization time was shortened to15 d, the vegetative stage of the plants might be prolonged by 10-15d in spite of its flowering and fruiting. The reason requires further research.

The results of the experiment showed that the germination rate and transplanting survival rate of immature embryos of winter wheat was greatly influenced by the embryonic age and types of medium. Yet the result of univariate analysis showed germination rate and transplanting survival rate of immature embryos of different genotype materials on the same medium varied slightly. In other words, the influence of embryonic age and medium is great but that of genotype is little. This conclusion is consistent with the research results of Ye et al. (1998).

Conclusions

This study demonstrates that the appropriate time for vernalization of seedlings was 20-25d; Among the nine inoculation tests, higher germination rate (95.80% on average) and transplanting survival rate of seedling (86.95% on average) were obtained from the 14-16d immature embryos cultured in the media without hormones, and the seedling emergence rate was the highest (82.95% on average). When seedlings were transplanted to the groove in summer field, we found the transplanting survival rate was greatly improved by covering the roots of seedlings with a small amount of detritus substrates and burying in soil. In summary, we have identified and optimized critical factors that influence the seedling emergence from immature embryos of winter wheat and their transplanting survival rate in summer field.

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