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Effect of ^{137}Cs Gamma Rays to Panicles on Rice Anther Culture

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Abstract: Panicles of an indica rice line TM7-5 were subjected to radiation with ^{137}Cs gamma rays at 0 (control), 5, 10, 15 and 20 Gy respectively, and then its anthers were cultured. There were slight differences among the treatments in peak emerging time of callus initiation, from 38 to 44 days after inoculation (DAI) as well as the frequency of callus initiation (2.3-3.5%). About two thirds calli were induced before 44 DAI, and calli derived beyond 60 DAI lost the regeneration ability. Green plant regeneration frequency was significantly stimulated from two- to three-fold by irradiation of the ^{137}Cs gamma rays compared with the control, and the maximum was 22.81% (15 Gy). The culture ability based on callus initiation and green plantlet regeneration was 0.19% for the control while it was over 0.45% for all the irradiated treatments, and the maximum was 0.59% for 15 Gy treatment. The advantages of panicle irradiation before anther culture and the potential application in rice anther culture, especially for recalcitrant indica rice, were discussed.

Key words: anther culture; gamma ray; panicle; radiation; culture ability; rice (*Oryza sativa*)

Anther culture plays an important role in rapid development of homozygous lines and genetic manipulation, especially in isolation of desirable individuals from segregating population in the early generation. The production of new varieties via anther culture shortens the breeding cycle, raises the efficiency of selection, and saves space and labor in the experimental field. For these reasons, intensive research on rice anther culture has been carried out since 1968, when Niizeki and Oono firstly regenerated plantlets from rice anther culture [1]. At present, along with continuous improvement in the techniques, anther culture has been widely combined with conventional approaches for japonica, as well as indica and hybrid rice breeding in China [2, 3].

There are several strategies to further increase the breeding efficiency, including improvement of anther-derived regeneration frequency, especially that of the recalcitrant varieties, combination of mutagenesis with anther culture and introduction of molecular marker assistant selection. In vitro irradiation is considered as a powerful method for rice improvement because gamma ray at suitable dose not only stimulates callus initiation and plantlet regeneration but also induces some mutation. Zhao et al [4] irradiated anther-derived callus with gamma ray and compared the effect of irradiation dose on regeneration frequency in 1983, they found that when the dose was above 6 kR the regeneration frequency would decrease sharply. Chen et al demonstrated that the dose of 20 Gy gamma ray had significant stimulation effect on regeneration of green plants from rice anther culture [5]. Nakamura and Hattori investigated the effect of ^{60}Co gamma-ray irradiation at different culture stages on rice anther culture [6]. Kinoshita et al [7] irradiated anthers just after inoculation on the callus-forming medium or anther-derived calli with gamma rays at acute or chronic irradiation rates. They found that the rates of green-plant regeneration were reduced under different irradiation treatments, except that from irradiated calli at the 500 R/min. Based on these results, in vitro mutagenesis of rice were used for selecting mutants with drought tolerance [8], salt tolerance [9], herbicide resistance [10], increasing contents of specific free amino acids [11, 12]. This method was also applied for anther culture of wheat, maize, oilseed, tomato, tobacco, petunia and apple etc.

However there are some disadvantages in irradiation of anthers just after inoculation on the callus-initiation medium or anther-derived calli. Firstly, medium suffered from irradiation will produce some toxin substance which will be harmful to either anthers or calli, so that they have to be immediately transferred to fresh medium after irradiation. Secondly, anther-derived calli consist a mixture of haploid and double haploid cells, while radiation-induced mutation will make the homozygous diploid calli return back to heterozygous ones again, and in this case the advantage of anther culture for breeding will be lost. We developed a new radiation way for anther culture that whole panicles were irradiated before anthers were inoculated on callus initiation medium. Here we report the results and the potential application of this method in breeding will be discussed.

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MATERIALS AND METHODS

An indica rice line TM7-5 was used for the experiment. Seeds were sown in May 2003 in the nursery, and the seedlings were transplanted in the experimental field at China National Rice Research Institute, Hangzhou, China.

Panicle treatment

Rice panicles at the late uninucleate pollen development stage were collected in the morning. Outer leaves were removed and the boots were cleaned with 75% ethanol, then wrapped in moist cheese cloth and polyethylene film and subjected to cold pre-treatment at 7℃ for 5 days. Then the panicles were irradiated with ^{137}cesium (^{137}Cs) gamma rays at the Institute of Crop and Nuclear Technology Utilization, Zhejiang Academy of Agricultural Sciences, Hangzhou, Zhejiang, China. The irradiation doses were 0, 5, 10, 15 and 20 Gy, respectively, all the treatments were finished within 30 minutes, so the dose rates were 0, 0.17, 0.33, 0.50 and 0.67 Gy/min. Anther culture was done within 10 hours after irradiation treatment.

Anther culture

Spikelets with microspores at mid- to late-uninucleate stage were selected from the radiated panicles and non-irradiated ones (control) respectively and were surface disinfected by immersing in 75% ethanol, then in 1.0% Antiformine for 20 min and in 3% hydrogen peroxide (H_2O_2) for 10 min, and finally the panicles were washed 3-4 times with sterile distilled water.

Anthers were inoculated on callus induction medium in 6-cm size Petri dishes, approximately 135 anthers per dish, 35 Petri dishes per treatment and incubated at 25±1℃ under dark conditions. The observation of callus formation was done every 5 days and the numbers of calli initiated were recorded until the 71st day after inoculation (DAI). Calli with the size of about 1-2 mm in diameter were transferred to the regeneration medium, about 15 calli per dish and then incubated at 25±1℃ under illuminated conditions (13.6 μ mol/m^2 • s, 15 h light / 9 h dark). The regenerated green shoots were transferred to the rooting medium in the test tube with 3 cm in diameter and 15 cm in length, one plantlet per tube and only one plantlet was selected from each callus. Plantlets were incubated at the same temperature and illuminated conditions as for plant regeneration.

Finally the plantlets about 10-cm tall with strong roots were removed from the tubes and directly transplanted to soil in the greenhouse.

Culture media

Rice anther culture media were based on our previous study [3]. The following media were adjusted to pH 5.8 with 1 mol/L KOH, prior to being autoclaved at 121℃ for 15 min.

Callus initiation medium was composed of M8 macro-elements, Fe-EDTA and vitamins [13], MS micro-elements [14], phenylacetic acid (PAA) 15 mg/L, naphthalene acetic acid (NAA) 2 mg/L, 6-benzylaminopurine (6-BA) 0.5 mg/L, casein enzymatic hydrolysate (CH) 500 mg/L, L-glutamine 500 mg/L, proline 100 mg/L, silver nitrate ($AgNO_3$) 7.5 mg/L, maltose 54 g/L and agar 8 g/L.

Regeneration medium was made up of MS macro- and micro-elements [13], M8 Fe-EDTA and vitamins [14], NAA 0.5 mg/L, 6-BA 2 mg/L, CH 500 mg/L, L-glutamine 500 mg/L, proline 100 mg/L, maltose 30 g/L and agar 8 g/L.

Rooting medium was contained with half concentration of N6 inorganic salts [15], sucrose 20 g/L and agar 8 g/L.

RESULTS

Effect of irradiation dose on callus induction

The inoculated anthers started to change their color from yellow to brown after five days of incubation, but there were about half of the inoculated anthers which all along kept the original yellow color and did not change color. Further observation indicated that those yellow anthers were not able to produce any callus.

The earliest two calli were found in 10 Gy treatment on 20 day after inoculation (DAI). Then the calli were observed from treatments of 0 Gy (control) and 5 Gy respectively on 22 DAI. As the irradiation dose increased, callus induction became slower, the first calli were detected on 24 and 26 DAI for 20 Gy and 15 Gy treatments respectively.

The frequencies of callus initiation among different treatments were compared in Table 1. That of the control (0 Gy) was 2.38%, and that of irradiation treatments ranged from 2.28% (10 Gy) to 3.50%(5 Gy). It is interesting that although the earliest calli emerged in the 10 Gy dose treatment, the callus induction frequency was the lowest.

Analysis of callus initiation kinesis showed that 18.28-21.25% of calli were induced before 30 DAI for 5, 10 and 20 Gy treatments, a little higher than the control (15.53%), while it was only 5.08% for 15 Gy treatment, significantly lower than the others. About two thirds calli were induced before 44 DAI, and the calli number were peaked during 38-44 DAI (Fig. 1) .

Effect of irradiation dose on plantlet regeneration

Calli were successively transferred to regeneration medium after 35 DAI, and the green plantlets or albinos were regenerated after 5-7 days. However, we found calli transferred to regeneration medium after 60 DAI reduced the regeneration ability with the increasing of incubation days, this phenomenon was more obvious in the control.

The frequency of green plant regeneration was calculated for each treatment (Table 1). It was clear that ^{137}Cs gamma ray irradiation significantly stimulate the frequency by two- to

Table 1. Effect of gamma irradiation dose on anther culture ability of indica rice (Rice line: TM7-5).

Radiation dose (Gy)	Anther number	Callus number	Callus initiation rate (%)	No. of calli for regeneration	No. of regenerated plants	Rate of plant regeneration (%)	Culture ability (%)
0	4320	103	2.38	88	11 (7)	12.50 (7.95)	0.19
5	3375	118	3.50	111	17 (15)	15.31 (13.51)	0.47
10	3510	80	2.28	74	23 (16)	31.08 (21.62)	0.49
15	2295	59	2.65	57	19 (13)	33.34 (22.81)	0.59
20	3510	93	2.57	87	19 (15)	21.84 (17.24)	0.46

Callus initiation rate: Number of initiated calli / Number of incubated anthers×100%;

Regenerated plant number: Total regenerated plants including albino, the numeral in brackets are green plantlets;

Rate of plant regeneration: Number of regenerated plantlets from callus / Number of incubated calli for regeneration × 100%, the numeral in brackets are rate of green plantlet regeneration;

Culture ability: Number of green plantlets / Number of incubated anthers × 100%.

three-fold, in addition the results also showed that there was an increasing trend of green plantlet regeneration frequency with the increasing of irradiation doses from 0 Gy (7.95%) to 15 Gy (22.81%), then a 5.57 percent point decrease was observed when the dose increased up to 20 Gy, but it was still about 10 percent point higher compared with the control. It suggested that we can obtain more plantlets if the regeneration medium is further improved. Table 1 also showed the increasing tendency of albino frequency as the irradiation dose raising, the highest one (10.53%) also existed at 15 Gy treatment.

The results revealed the culture abilities (number of green plantlets / number of incubated anthers × 100%) of all the treatments were higher than 0.46%, twice above the control (0.19%). The highest culture ability (0.59%) was also observed at 15 Gy treatment (Table 1).

DISCUSSION

From the view of breeding, high regeneration frequency of green plantlets is the prerequisite for successful application of the anther culture technique on rice breeding. However, anther culture is strong genotype dependent, especially for indica, which limits its wide application in rice breeding. Biotechnologists have tried their best to overcome this problem for 30 years and made successes. One way is in vitro gamma irradiation with lower dose, which was considered a powerful way due to combine the characters of anther culture and mutagenesis, the common method is irradiation on anthers just after inoculation at callus-initiation medium or anther-derived calli. We developed a new method, i.e. direct irradiation of rice panicles before anther inoculation and the culture ability increased from 0.19% (without irradiation) up to 0.59% (15 Gy). It seems to be several advantages compared with previous methods. Firstly, panicles is easier to be operated than the Petri dishes containing anthers. Secondly, direct irradiation saves more labors and time because they just follow the normal protocol and does not need any extra work, while the radiated anthers or calli on medium have to be transferred to new Petri dishes immediately after irradiation in order to avoid the toxin in the medium derived by gamma rays.

The culture ability is the most important parameter in rice anther culture. The previous studies indicated that rice plant regeneration frequencies increased significantly after callus radiation [4, 5]. In this experiment we found that although the

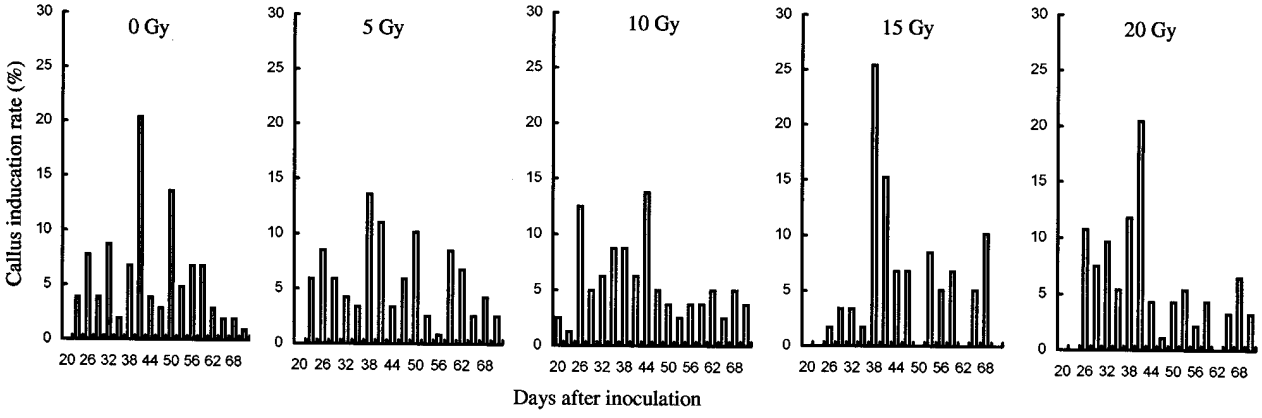

Fig. 1. Kinesis of callus induction rate based on callus number at a given period to the total calli derived from rice anthers on callus initiation medium.

callus initiation frequency did not increase after irradiation, the regeneration frequency of the radiation treatment was much higher than that of control. It is reasonably conjectured that gamma ray could stimulate callus regeneration by some mechanism such as activation of retrotransposome [16]. Higher culture ability makes application of panicle radiation be possible both for breeding and mutation induction. It is another benefit for irradiation of rice panicles that any potential mutation induced by gamma rays will be homozygous in anther-derived plantlets, while the mutation induced at radiated calli will make the regenerated plantlets heterozygous.

Although the anther culture ability increased in this experiment, and the highest is 0.59%, there still far lower than that of japonica rice which is over 1% in general. There are still several rooms to be improved. One is to increase the response ability of anthers to in vitro culture. Color change from yellow to brown is the first visible reaction of rice anther to in vitro culture, however there were about 30% anthers without color change during cultivation, it was even found that the whole anthers in several Petri dishes did not change their color. Besides, there were 5-10% green spots did not differentiated whole plantlets, it suggested that medium improvement and donor plant growth condition change may be first considered in the future.

The experiment is still continuing for further observation on frequency of DH plants and the mutation frequency.

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