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SEASONALITY OF ANDROGENETIC RESPONSES IN THE ANTHER CULTURE in vitro IN RICE (*Oryza sativa* L.)

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Abstract

The conditions for growing donor plants in androgegesis in vitro are considered from the point of view of the influence of physical factors on the plants (illumination, length of daylight, temperature, nutrition of plants). A priori it is believed that any period of the year (season) is suitable for plant tissue culture in vitro, and this is the main advantage of in vitro technologies compared to traditional ones. Seasonality is taken into account only when comparing donor plants grown in the field and under controlled conditions. However, we have not found reports on the study of using procedure of anther in vitro culture throughout the year. This paper is the first to show that, under uniform conditions of donor plants growing, the frequency of callus formation and regeneration of rice (*Oryza sativa* L.) plants from anthers in vitro culture differs depending on the month (season) when explants were collected. The aim of the studies was to study the seasonal dependence of in vitro androgenetic responses of *O. sativa* when growing donor plants in a climatic chamber conditions. The 5-9 plants of *O. sativa* subsp. *japonica* Kato, the Cascade variety, were planted monthly during a year and grown in a climatic chamber (at 24 °C and 21 °C, 15000 lux, 60 % humidity and photoperiod of 14 light/10 dark hours) to be the test anther donors. The anthers from naturally grown plants served as controls. As a result, the seasonal dependence of callus formation in the rice anther culture in vitro was revealed. The peak intensity of callus formation occurs in May and June (15.5-28.3 %). When growing donor plants under artificial conditions at both temperatures, in the best period for anther culture in vitro (May-June) callus formation was higher than in the control. The use of the climatic chamber makes it possible to obtain consistently high values of the intensity of callus formation in some months, which is not always possible in the natural conditions. The temperature of donor plant growing affects the frequency of rice callus formation and the regenerative capacity. The temperature which is lower than optimal for rice plants (21 °C) allows for different frequency of callus formation throughout the year, whereas the rice-comfortable temperature (24 °C) leads to a large seasonal dependence and callus formation from May to September and in December and January. At 21 °C vs. 24 °C, four times more calluses with green regenerants are formed (31.6 % vs. 8.8 %), with an increase in the fraction of doubled haploids up to 28.1 % and their number per callus up to 16.6. A moderate correlation was found between the share of calli with green regenerants and the average number of doubled haploids per callus ($r = 0.59$ at $p = 0.05$). This means that with an increase in the number of calli with green regenerants, which is observed at 21 °C, the total yield of doubled haploids also increases. As to the number of haploids, no such dependence was found. Thus, greenhouses and climatic chambers may serve not only for growing donor plants by researchers, but also for practical use of the most favorable periods for anthers culture technique.

Keywords: *Oryza sativa* L., androgenesis in vitro, callus formation, regeneration, doubled haploids, seasonality

An in vitro rice anther culture has been used for genetic researches and breeding purposes since 1968 [1]. The works for obtaining doubled rice haploids has been successfully carried out for five decades, however, the breeding of new varieties, hybrids, and lines requires the optimization of anthers cultivation conditions because a significant dependence of callus formation and regeneration on the parent plant's genotype is known. It is considered a key problem in the

plants androgenesis *in vitro* including that in rice [2-4]. Nevertheless, there are also a number of other factors which influence on the androgenesis process: the conditions of donor plants growing, shock treatment of anthers before introduction into the *in vitro* culture, compositions of culture media and conditions of anthers and calli culturing [5-7].

The conditions of donor plants growing are considered from the standpoint of their exposure to the physical factors (light intensity, daylight duration, temperature, plants nutrition) [5, 8]. It is believed that any period of the year (season) is suitable for the in vitro culture of the plants' cells and tissues that constitutes the main advantage of this technology compared to traditional agricultural production [9]. However, experienced researchers know that in certain periods of a year in vitro cultures grow and develop better than in other ones. Some rare experimental data on the plants' seasonal development in the *in vitro* conditions began to appear. Thus, the propagation by cutting of the *Sequoiadendron giganteum* (Lindl.) J. Buchholz in spring (March-May) contributes to the longer preservation of more number of living cuttings [10]. The summer period was the most favorable for the essential oil rose grown in the foothill zone of the Crimea. In this period, the number of developing explants in five varieties reached 92-97%, and in autumn this value was minimal, 40-72% [11]. For roses grown on the southern coast of Crimea the best period for selecting and introducing the explants into the culture were February-March, when the meristems development frequency reached 92-100%, in the autumn-summer period this value did not exceed 10-20% [12]. The authors conclude that such morphogenetic reaction is conditioned by the physiological state of the plant's organ and of the explant secreted from it [12]. We have not found any reports related to the anthers introduction in the in vitro culture in all periods of the year. The seasonality factor is considered solely when comparing the donor plants grown in the field and indoor conditions.

Some researchers introduce the anthers in the in vitro culture year-round without any stable result, but in most cases they try to time the cultivation by the end of spring. There are many information releases about using greenhouses and climate chambers for growing the donor plants for the rice anther culture [13, 14]. The main advantage of using indoor growing is the possibility to regulate the growing conditions [5, 6] and the aseptic purity of anthers introduced into the culture [5, 15]. In all such works, the emphasis is laid on the parent plants' growing conditions and not on the seasonality.

In the consideration of the problems related to the donor plant growing conditions there is also no the clear-cut answer to the question how to get the maximum output of doubled haploids. The relevant researchers are unanimous in the opinion that the conditions must be different for different species, genera, and families [4, 16]. In most cases, the researches try to create the maximally favorable conditions for the growth and development of the parent plants of rice [2, 6] both in the field [17-19] and indoor [13, 14] conditions.

There are some evidences that the stressful conditions of the parent plants growing favorably influence the callus formation or shoots regeneration processes. In the anthers of the rice plant growing in a dry season there are more microspores capable of androgenetic responses [6]. In the plants grown at 18-20 °C, the frequency of calli production and regeneration of green shoots were more than 2 times higher than when growing at 26-28 °C, and the albinos formation was lower [as cited in 20]. The frequency of green regenerants formation turned out to be higher when growing the donor plants in the culture room at 20 °C than when growing in open space [21]. All these experimental data have also been obtained regardless of the seasonal dependence.

This research for the first time showed that the intensity of the callus formation and regeneration of rice plants varies depending on the month when the anthers were introduced in the in vitro culture while the donor plants' growing conditions were uniform throughout the year. The most favorable period for the introduction of anthers in the in vitro culture was May-June. The lower temperature of 21°C made it possible to obtain callus with different intensities throughout the year, and when the temperature of 24 °C which is optimal for rice, the higher seasonal dependence was observed.

The objective of this work was to investigate the seasonal dependence of the androgenetic responses of rice plants *Oryza sativa* L. when the donor plants growing *in vitro* in the controlled conditions of climatic chamber under two temperature schedules.

Techniques. The anthers of rice *O. sativa* subsp. *japonica* Kato Cascade variety were used in the researches (Chaika Far East Federal Research Center for Agrobiotechnology). The 5-9 donors planted in the ground on Day 15 of every month of the year were placed into the climatic chamber MLR-352H (Sanyo, Japan) under the following conditions: temperature 24 °C (in 2015) and 21 °C (in 2016), light intensity 15,000 lux, humidity 60%, photoperiod (light/dark) 14/10 hours. Donor plants grown in pots in the open space were the control. In the control, anther cultures were derived from 400 anthers in August 2015 and 2016.

Rice anthers prior to culture were exposed to low positive temperatures (5 °C) for 7 days by placing the panicles into the cylinders with water. For culture, induction culture medium N_6 was used [2]. A total of 400 to 702 anthers per month were used.

The anthers were cultured at 25-27 °C in the dark until the 1-5 mm calli formation. Then it was transplanted to the N_6-рк medium [23] for the shoots regeneration. The conditions in the culture room were the following: light intensity 4,000 lux, temperature 22-25 °C, daylight/dark duration 16/8 hours. For the rooting the MS medium [24] with the half-amount composition of macrosalts was used as modified by Goncharova [20].

The regenerants with a developed root system were planted in pots and continued to grow in the culture room until the seeds formation. All regenerated plants were divided into groups of haploids (the plants without seeds and with very small flowers), doubled haploids (the plants with seeds), tetraploids (the plants with few very large seeds having the apparent keel and ribbing on the floral squames), the plants without seeds (which have formed flowers of normal size, but not seeds on two or more panicles), and the plants which died during early growth.

The data statistical processing was made using Statistica 10 software (StatSoft Inc., USA). The mean values (M), standard errors of means (±SEM) and the correlation coefficient (r) were determined. The difference between the variants was evaluated by Student's t-test at the significance level of at least 5%.

Results. For 24 °C and 21 °C, 5,172 and 7,278 anther cultures were initiated, 12,450 in total. The seasonal dependence was detected at both temperatures of the parent plants growing; the maximum frequency of callus formation occurred in May under 24 °C (Fig. 1, A) and in May-June under 21 °C (see Fig. 1, B). However, the temperature which is low for the rice plant made it possible to get the callus every month with more or less intensity (1.0-15.5%). At the optimum temperature of 24 °C in the early spring and late autumn periods, the callus formation did not occurred. The monthly callus formation averaged to 4.5% for 24 °C and to 3.9% for 21 °C without statistically significant differences.

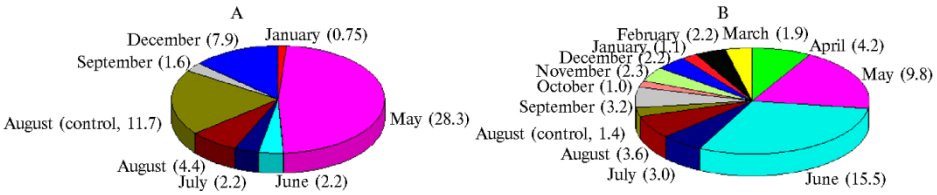

Fig. 1. Callus formation (%) in rice (*Oryza sativa* spp. *japonica*) Cascade variety depending on the month of anther culture initiation and on the temperature of growing donor plants: A — 24 °C, B — 21 °C.

1. Calli with green and albinos regenerants in rice (*Oryza sativa* spp. *japonica*) Cascade variety depending on the month depending on the month of anther culture initiation and on the temperature of growing donor plants

Month	Call, %		Green regenerants/albinos
	with green regenerants	albinos	
Temperature 24 °C			
May	13.7	37.1	3.5
June	7.7	23.1	20.5
July	5.9	26.5	0.8
August (control)	12.8	25.5	1.3
August	5.6	16.7	0.9
September	0	33.3	0
December	15.9	15.9	24.3
Average (*M*±SEM)	8.8±2.1	25.4±3.0	7.3±3.9
Temperature 21 °C			
January	33.3	16.7	20.0
February	21.4	21.4	23.7
March	50.0	16.7	18.3
April	20.0	20.0	7.6
May	33.3	22.2	18.4
June	56.3	59.8	5.4
July	23.1	7.7	9.8
August	33.3	52.4	2.3
August (control)	0	40.0	0
September	22.2	38.9	8.0
October	60.0	20.0	44.0
November	50.0	16.7	21.1
December	20.0	26.7	50.0
Average (*M*±SEM)	31.6±5.0	27.6±4.3	17.6±4.2

Note. The mean values (*M*) of the share of the calli with green regenerants for 24 °C and 21 °C differ statistically significantly at $p = 0.01$.

The callus frequency in the control was different in 2015 and 2016. Thus, the conditions of 2015 were typical for parent plants' growth that led to the higher callus formation (11.7%) (see Fig. 1, A). In 2016, we collected panicles in hot period that could be a negative stressor for the callus formation which amounted to 1.4% (see Fig. 1, B), and led to the complete absence of shoot regeneration. Under growing donor plants in a chamber at both temperatures during the best time of the anther culture initiation (May-June), the callus formation was higher than in the control. Thence, using the climatic chamber made it possible to get a stable high callus formation in certain months, which is not always possible under the natural conditions of growing anther donors.

Donors grown under 24 °C produced 8.8% calli capable of green regenerant formation, at 21 °C the average value was 4 times higher ($t = 3.35$, $p = 0.004$), up to 31.6 % (Table 1). Therefore, the temperature which is low for rice plant not only allows callus formation throughout the year, but also influences the frequency of calli with morphogenetic responses. There was tendency to a 2-fold decrease of albinism. Note that when using other methods to increase shoot regeneration from anthers, e.g. different regeneration media, the number of green regenerants also increases due to the greater output of calli capable of morphogenetic responses [23].

Albinism of calli is a problem for plant regeneration in cereal crops [4, 6]. In our research, the albinos were several times less frequent than green regenerants (see Table 1); they appeared only at the initial stage of morphogenesis and could be easily removed from the callus. The next passage of callus aggregates with green initials allowed tens and hundreds of green regenerants to develop normally.

2. The average number of rice (*Oryza sativa* spp. *japonica*) Cascade variety green regenerants per callus depending on the month of anther culture initiation (M±SEM)

Month	Calli	Haploids	Doubled haploids	Tetraploids	Dead plants	Seedless plants
Temperature 24 °C						
May	17	54.1±13.3	14.8±3.9	0	11.2±3.5	0.3±0.2
June	1	157	4	0	44	0
July	2	16.0±10.0	13.5±9.5	0	2.5±1.5	0
August (control)	6	17.2±15.4	3.7±1.8	0	1.7±0.8	0
August	1	14	0	0	1	0
December	7	54.9±24.4	28.0±11.3	1.6±1.6	11.1±8.9	1.4±0.8
Average	5.7±2.5	10.9±5.6	4.6±2.4	0.3±0.3	11.9±6.7	0.3±0.2
Temperature 21 °C						
January	2	90.5±0.5	1.0±0.0	0	8.5±0.5	0
February	3	33.0±31.5	28.3±27.3	0.3±0.3	9.7±2.9	4.3±4.3
March	6	17.3±9.4	23.5±17.9	0	14.2±7.8	0
April	5	27.4±13.3	9.6±5.6	0	9.8±7.2	0.2±0.2
May	17	31.9±8.4	19.7±6.9	0.5±0.5	5.4±1.0	1.9±1.2
June	52	28.0±5.4	10.9±2.5	0.3±0.3	8.9±1.4	2.4±0.6
July	3	3.7±2.0	9.7±7.3	0	3.0±2.1	0
August (control)	7	21.3±13.4	5.9±2.4	0	4.1±1.8	1.4±1.0
September	4	34.0±21.1	18.5±11.2	0	9.8±1.4	1.0±0.6
October	3	3.3±1.3	61.7±30.9	0	7.0±3.4	1.3±0.7
November	6	12.0±5.7	12.3±7.0	0	3.3±3.3	0.8±0.4
December	3	51.7±24.1	15.0±10.0	0	15.0±9.0	0
Average	9.3±4.1	27.2±6.7	16.6±4.3	0.1±0.1	8.2±1.1	1.1±0.4

Note. The average values for doubled haploids at two temperatures differ statistically significantly at p = 0.05; the differences in mean values of the haploids number are statistically insignificant.

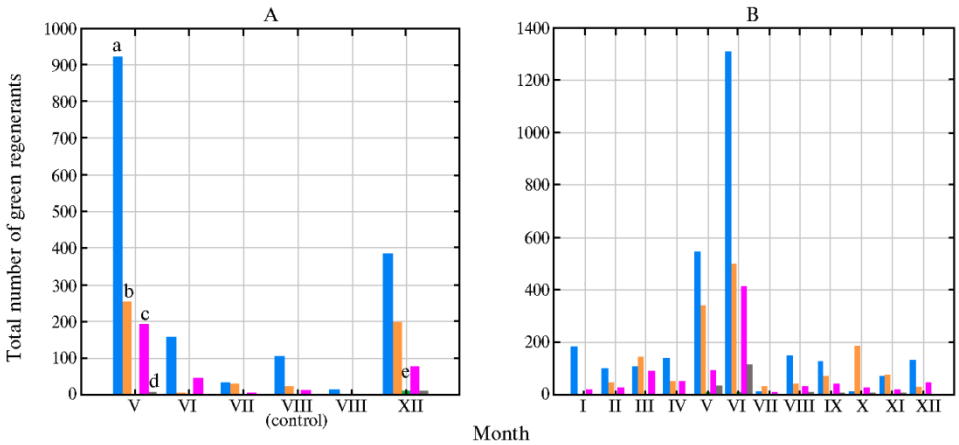

Fig. 2. Regeneration of rice (*Oryza sativa* spp. *japonica*) Cascade variety depending on the month of anther culture initiation for donor plants growing temperatures of 24 °C (A) **and 21 °C** (B)**:** a — haploids; b — doubled haploids; — dead plants; d — plants without seeds; e — tetraploids.

The average amount of regenerants of various types per callus depending on the temperature of donor plant growing is shown in Table 2. There were no statistically significant differences between haploids and doubled haploids as per months. The statistically significant difference (t = 4.26, p = 0.0001) was only between the average values of doubled haploids in June and October for 21 °C of growing donor plants. The average values of doubled haploids differed (t = 2.39, p = 0.05) between 24 °C and 21 °C temperatures (see Table 2). There was moderate correlation between the portion of the calli with green regenerants and the

average number of doubled haploids per callus (r = 0.59, p = 0.05). It means that as an increase in the number of calli with green regenerants occurred at 21 °C, the total output of doubled haploids also increased. Such dependence by the number of haploids has not been revealed.

For 24 °C and 21 °C the total number of produced regenerants amounted to 2,464 and 5,798, respectively. The maximum number of regenerants was in May-June period in accordance with the callus formation intensity (Fig. 2). For 24 °C, a regenerative ability slightly increased in December.

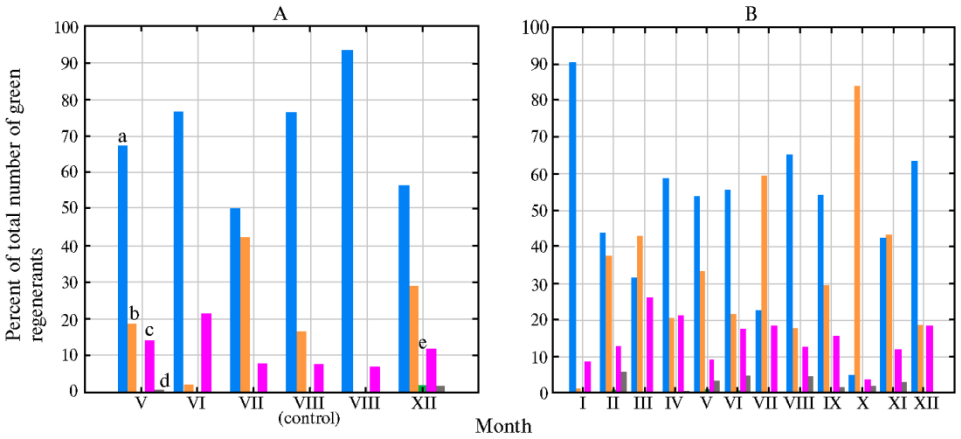

Fig. 3. Groups of regenerants of rice (*Oryza sativa* spp. *japonica*) Cascade variety depending on the month of anther culture initiation for donor plants growing temperatures of 24 °C (A) **and 21 °C** (B)**:** a — haploids; b — doubled haploids; — dead plants; d — plants without seeds; e — tetraploids.

The number of regenerants of different types was not the same for different temperatures of growing donor plants. At 24 °C, haploids were always the greatest in number, throughout the year up to 65.3% and from 50.0 to 93.3% as per months (see Fig. 3, A). Doubled haploids amounted 20.3% on average per year and varied from 0 to 42.2% as per months. At 21 °C, throughout the year haploids averaged 52.6%, ranging from 4.6 to 90.5%, and doubled haploids reached 28.1%, ranging from 1.0 to 84.1% (see Fig. 3, B). That is, as the temperature used to grow donors decreased, the number doubled haploids increased. This was due to the increase in the average number of regenerants from callus. In certain months (March, July, October and November), if the donor plants were exposed to lower temperature (21 °C), doubled haploids prevailed over haploids (see Fig. 3).

No dependence has been found for nonviable regenerants, tetraploids, and sterile plants of non-haploid origin. Seasons of the year and the temperature of the donors growing did not affect the frequency of their appearance. Apparently, their formation in callus was spontaneous.

The found seasonal dependence of androgenetic responses in rice is well consistent with the general biological laws. Spring and early summer is the most favorable period for active germination of seeds and vegetative propagation of plants. Obviously, even under controlled growing donors, callus formation in anther culture and regeneration intensify precisely in this period. The propagation by cutting of other plants in vitro is also most successful in spring [10-12]. Indoor growing (in greenhouses, climatic chambers) not only ensures the controlled conditions for growing donor plants, which is often practiced by researchers [5, 6], but also enables using favorable time to initiate anther culture. Pershina et al. [25] note the negative response of some wheat varieties in anther culture when growing donor plants in a greenhouse. Perhaps, given the seasonal

dependence of androgenic responses, the result could be positive.

The low temperature during the donor plants growing (21 °C) is an additional stressor which stimulates the androgenetic responses of the rice plants and made it possible to get the regenerants throughout the whole year, while more favorable temperature (24 °C) is effective only during certain months. Shock temperatures (4-12 °C) of anther pretreatment can switch the program of microspore development in cereals from gametophytic to sporophytic [2, 5, 7]. The extremely low temperature of donor growing enhances this effect in rice. A number of researches show that creating the better conditions for the donor plants' growth and development is not necessary as it is commonly believed. A stress including the temperature stress enhances the callus formation and regeneration [as cited in 20, 21].

Thus, there is the seasonal dependence of callus formation in rice anther culture, with the maximum frequency in May to June. The temperature of donor plant growing influences callus formation intensity and regenerative ability. The temperature which is lower than the optimal for the rice plants (21 °C) ensures callus formation with different intensity throughout the whole year, while at the optimal temperature (24 °C) the callus formation is possible only from May to September and in December to January. The anthers of donor plants grown at 21 °C produce practically 4 times more calli with green regenerants (31.6%) as compared to those at 24 °C (8.8%). The number of doubled haploids increases up to 28.1% and averages up to 16.6 per callus.

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