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STUDIES ON THE ABNORMALITY OF EMBRYO SAC AND POLLEN FERTILITY IN AUTOTETRAPLOID RICE DURING DIFFERENT GROWING SEASONS

MUHAMMAD QASIM SHAHID[1], SUN JIANFAN[1], WEI CHANGMIN[1], ZHANG PENG[1] AND LIU XIANG-DONG[1*]

[1]*Guangdong Provincial Key Laboratory of Plant Molecular Breeding, South China Agricultural University, Guangzhou, Guangdong, 510642, China.*

Abstract

Autotetraploid rice has a great genetic potential but low seed setting rate is the major encumbrance in its use. Embryo sac fertility and pollen fertility are the most important factors which affect the seed setting rate in autotetraploid rice. Whole mount eosin B-staining confocal laser scanning microscopy (WE-CLSM) was used to study the fertility and abnormalities in embryo sacs of diploid and autotetraploid rice during different seasons. The results indicated that the embryo sac fertility (64.5%) was much low in autotetraploid than that in diploid rice (86%), and five main types of abnormal embryo sac were found in all 10 autotetraploid rice. Moreover, some other type abnormal embryo sacs were also observed in autotetraploid rice. Embryo sac without female germ unit and embryo sac degeneration were the most frequent types of abnormalities in autotetraploid rice. Embryo sac fertility ranged from 49.3% to 79.3%, pollen fertility ranged from 56.2 to 85.9%, and seed setting rate varied from 12.5 to 69.01% in various genotypes of autotetraploid rice. Embryo sac and pollen fertility were found to have a significant correlation with seed setting rate. Seasons have significant effect on pollen and embryo sac fertility in both type of rice. All the autotetraploid lines exhibited different types of embryo sac abnormalities which indicated that these might be related to different genotypes.

Introduction

Rice is one of the most important cereal food crop which provides food to more than half of the world's population (Takashi *et al.,* 2005). Rice is the second staple food crop of Pakistan (Ahmad *et al.,* 2008), and plays an important role in the economy of Pakistan and major export commodity of country (Akram *et al.,* 2007; Rashid *et al.,* 2007). There are mainly two distinct growing seasons of rice in tropics, temperature and precipitation played an important role in biomass production and yield during these two seasons (Laza *et al.,* 2003). A significant decrease in the spikelet fertility occurred when it was exposed to higher temperature (35°C, 38°C and 41°C) at flowering time or nine days before heading which caused pollen abnormalities in diploid rice (Satake & Yoshida, 1978). Kim *et al.,* (1996) revealed that high temperature (> 33°C) reduced the pollen production and pollen fertility which tend to decline the spikelet fertility. Exposure to high temperature during heading significantly reduced the spikelet fertility but different genotypes responded variably (Prasad *et al.,* 2006; Jagadish *et al.,* 2007). Polyploidy is an important source of crop improvement and it is a hard task to further increase the yield of crops through diploid breeding programs due to its small genome (Bingham *et al.,* 1994).

[*]Corresponding author: E-mail: xdliu@scau.edu.cn;
Phone: +86-20-85285752, Fax: +86-20-38294893

Polyploidy has played a significant role in the plant evolution and has attracted the attention of a number of scientists (Grant, 1971; Soltis *et al.,* 2004; Durand & Hoberman, 2006; Chen & Ni, 2006; Udall & Wendel 2006). Autotetraploid rice is a special genetic material which can overcome the stagnation in rice improvement. Polyploidy increases the resistance against insect pest and diseases, high biomass production and drought tolerance (Wendell, 2000). Autotetraploid has more genetic variation than diploid rice (Luan *et al.,* 2008), but low seed setting rate is a major barrier in the utilization of autotetraploid rice (Guo *et al.,* 2002; Li & Xu, 2000). Embryo sac fertility play significant role in the spikelet fertility of autotetraploid rice. However, it is difficult to observe the more number of embryo sac by conventional techniques such as plastic section or whole-stain clearing techniques because rice embryo sac wrapped by nucellus, integument and ovary wall (Zeng *et al.,* 2007, Guo *et al.,* 2006). WE-CLSM (whole mount eosin B-staining confocal laser scanning microscopy) was developed by our lab (Guangdong provincial key laboratory of plant molecular breeding, South China Agricultural University, Guangzhou, Guangdong province, China), which is easy to use and produced clear image in quick time (Zhang *et al.,* 2003, Zeng *et al.,* 2007). This study was conducted to observe the types of abnormal embryo sacs through WE-CLSM and effect of seasons and genotypes on autotetraploid lines including *japonica* and *indica* and their relationship with seed setting rate.

Materials and Methods

Ten diploid (CK) and their autotetraploid lines were used to investigate the embryo sac fertility and pollen fertility (Table 1). Autotetraploid lines are genetically stable and being cultivated in our lab from last ten years. All of these materials were cultivated at the farm of South China Agricultural University (SCAU), Guangzhou during early season (March-July, 2007), later season (July-Dec, 2007) and early season (March-July 2008). To observe the effect of different seasons, Embryo sacs (ES) were observed during first two seasons whereas pollen fertility (PF) was checked during three seasons. The heading dates ranged from 31 May to 24 June, 2 October to 28 October and 28 May to 22 June during early season 2007, late season 2007 and early season 2008, respectively. All the field and planting practices were kept according to the recommendation of area.

Embryo sac and pollen fertility analysis: The mature florets with open glumes were collected from 9:00 to 12:00 noon and then fixed according to Zeng *et al.,* (2007). The florets were rinsed in 70% ethanol and lemma and palea cut under the binocular dissecting microscope, ovaries was then exposed to series of ethanol (50%, 30% and 10%) and distilled water. WE-CLSM was done according to Zeng *et al.,* (2007) with some minor modifications. The ovaries were rinsed in 2% aluminum potassium sulphate for 30 min., and then stained with eosin B (10 mg/L dissolved in 4% sucrose solution) for 10-12 h at room temperature. The ovaries were treated again with 2% aluminum potassium sulphate for 30 min., which produced clear image by removing dye from ovary wall. The samples were washed thrice with distilled water and dehydrated by passing through a series of ethanol solutions 30%, 50%, 70%, 90% and 100% for 30 min. The dehydrated samples were subjected into a mixture of ethanol absolute and methyl salicylate (1:1) for 2-4 h and then cleaned for 1-3 h in pure methyl salicylate solution. The samples were placed on a slide and observed under a Leica SPE Laser scanning confocal microscope (Heidelberg, Germany). The direction of the embryo sac kept in a specific direction, so that the egg apparatus, polar nuclei and antipodal cells have maximum slice area. Four to six images of different focal lengths were saved.

Table 1. Diploid and autotetraploid cultivars-lines used in the study.

Code	Name	Ploidy	Origin/Source	Subspecies
DE	E24	2×	Guangdong, China	*japonica*
D2	Guanglu'ai 4	2×	Guangdong, China	*indica*
D3	L202	2×	IRRI	*japonica*
D5	Jackson	2×	IRRI	*japonica*
D23	Shengnong 265	2×	Shen Yang, China	*japonica*
D24	Shengnong 15	2×	Shen Yang, China	*japonica*
D28	Linglun	2×	Hunan, China	*indica*
D47	IR36	2×	IRRI	*indica*
D49	M18	2×	Guangdong, China	*japonica*
D51	Dali'nuo	2×	Guangdong, China	*indica*
TE	E24	4×	Lab	*japonica*
T2	Guanglu'ai 4	4×	Lab	*indica*
T3	L202	4×	SCBG- CAS	*japonica*
T5	Jackson	4×	SCBG- CAS	*japonica*
T23	Shengnong 265	4×	Lab	*japonica*
T24	Shengnong 15	4×	Lab	*japonica*
T28	Linglun	4×	Lab	*indica*
T47	IR36	4×	Lab	*indica*
T49	M18	4×	Lab	*japonica*
T51	Dali'nuo	4×	Lab	*indica*

IRRI: International Rice Research Institute; Lab: Guangdong provincial key laboratory of plant molecular breeding, South China Agricultural University, Guangzhou, Guangdong province, China; SCBG-CAS: South China Botanical Garden, Chinese Academy of sciences

About five to ten mature spikelets were collected from various panicles of the plant to investigate the pollen fertility, then fixed in FAA solution (50% ethanol: acetic acid: formalin with ratio of 89:6:5, respectively) for 24 h. Potassium Iodide solution (I_2-KI, 2%) was used to stain the spikelets and observed under microscope. Pollen fertility was divided into four categories i.e., normal pollens, stained abortive pollens, spherical abortive pollens and typical abortive pollens.

Statistical analysis: Spikelets were randomly selected from five plants of each cultivar. Seed setting rate (SSR) was calculated as mean of all plants. Analysis of variance was done by SAS (Anon., 2003) to compare the effect of season and ploidy on pollen fertility, embryo sac fertility and seed setting rate.

Results

Types of abnormal embryo sacs in autotetraploid rice during different seasons: All the diploid varieties (CK) mostly produced normal embryo sacs but it varied among autotetraploid lines from normal to abnormal embryo sacs (Table 2). Normal embryo sac contains one egg cell, two synergids at the micropylar end, a group of antipodal cells at chalazal end and two polar nuclei above the egg apparatus (Fig. 1A-B and 2A-B). Abnormal embryo sacs found in all diploid and autotetraploid rice lines were as follows:

1. Embryo sac degeneration (ESD): In this abnormal type of embryo sac whole embryo sac or cavity of embryo sac degrades (Fig. 1C and 2C).

Table 2. Frequency of abnormal types of embryo sacs in diploid and autotetraploid rice during early and late season 2007.

	Early season 2007							Late season 2007						
	ESD (%)	**ESWF (%)**	**EAD (%)**	**ESAP /ESAN (%)**	**SES (%)**	**OAT (%)**	**Total frequency of AES (%)**	**ESD (%)**	**ESWF (%)**	**EAD (%)**	**ESAP /ESAN (%)**	**SES (%)**	**OAT (%)**	**Total frequency of AES (%)**
Diploid														
DE	3.6	5.3	1.8	0.2	0.0	0.0	10.9*	2.7	3.3	0.9	0.7	0.2	0.4	8.2 **
D-2	1.1	2.2	0.9	0.4	0.0	0.0	4.6**	1.8	2.7	0.7	0.2	0.2	0.3	5.9 **
D-3	4.0	9.8	2.4	2.0	0.9	0.2	19.3**	3.3	4.7	1.6	1.1	0.1	0.6	11.4 *
D-5	4.9	4.6	3.4	0.4	0.0	0.0	13.3**	4.4	4.9	3.6	0.9	0.0	0.0	13.8 **
D-23	3.8	8.9	5.3	1.6	0.0	0.2	19.8**	6.7	4.4	4.9	1.8	0.0	0.5	18.3 **
D-24	5.6	4.2	1.3	1.8	0.0	0.0	12.9*	4.9	3.6	0.4	0.2	0.0	0.0	9.1 *
D-28	5.8	8.4	3.8	1.3	0.0	0.4	19.7*	3.3	4.7	3.6	2.4	0.0	0.0	14.0*
D-47	16.4	3.3	0.9	0.9	0.2	0.2	21.9	4.2	7.3	3.1	1.6	0.0	0.0	16.2 **
D-49	3.3	6.0	1.3	1.1	0.0	0.0	11.7 *	5.6	4.0	1.8	0.4	0.0	0.0	11.8 *
D-51	4.7	10.2	5.3	1.6	0.0	0.0	21.8*	7.1	3.3	4.7	2.0	0.9	0.6	18.6 *
Mean	**5.3**	**6.3**	**2.7**	**1.1**	**0.1**	**0.1**	**15.6 *^{s}**	**4.4**	**4.3**	**2.5**	**1.1**	**0.1**	**0.2**	**12.7**
Autotetraploid														
TE	8.7	10.0	5.2	4.5	1.3	2.7	32.4	6.8	9.3	5.0	3.2	0.6	0.9	25.8
T-2	5.7	11.8	4.5	3.0	1.5	1.8	28.3	4.3	10.2	3.2	1.3	0.8	1.4	21.2
T-3	11.8	9.5	3.7	4.2	2.3	3.2	34.7	9.8	5.3	4.7	4.2	1.6	2.1	27.7
T-5	8.3	8.5	2.5	8.2	1.0	3.6	32.1	11.7	11.2	4.0	7.6	0.5	1.6	36.6
T-23	13.2	17.5	11.3	1.2	4.2	3.8	51.2	19.2	14.0	6.7	7.4	2.4	1.2	50.9
T-24	10.2	19.8	6.7	2.5	1.8	1.0	42.0	11.2	9.7	5.8	3.5	2.7	4.3	37.2
T-28	18.2	12.0	8.7	6.5	1.3	1.1	47.7	11.3	7.5	6.7	5.3	3.1	3.9	37.8
T-47	11.2	13.0	1.8	2.4	1.4	2.1	31.9	10.3	5.5	2.7	2.6	1.5	1.3	23.9
T-49	11.3	23.2	6.3	2.9	2.8	1.3	47.8	11.8	7.8	6.0	4.3	2.3	2.8	35.0
T-51	14.2	16.7	5.0	5.5	1.9	6.2	49.5	15.5	11.0	6.3	5.8	3.0	4.5	46.1
Mean	**11.3**	**14.2**	**5.6**	**4.1**	**2.0**	**2.7**	**39.8 **^{s}**	**11.2**	**9.2**	**5.1**	**4.5**	**1.9**	**2.4**	**34.2**

*, ** Diploid and autotetraploid are significantly different at 0.05 and 0.01 probability level. *^{s} and **^{s} represent seasonal difference.

ESD, embryo sac degeneration; ESWF, embryo sac without female germ unit; EAD, egg apparatus degeneration; ESAP/ESAN, embryo sac with abnormal polar nuclei number or position; SES, small embryo sac; OAT, other abnormal types; AES, abnormal embryo sacs.

150 embryo sacs were observed for each cultivar-line

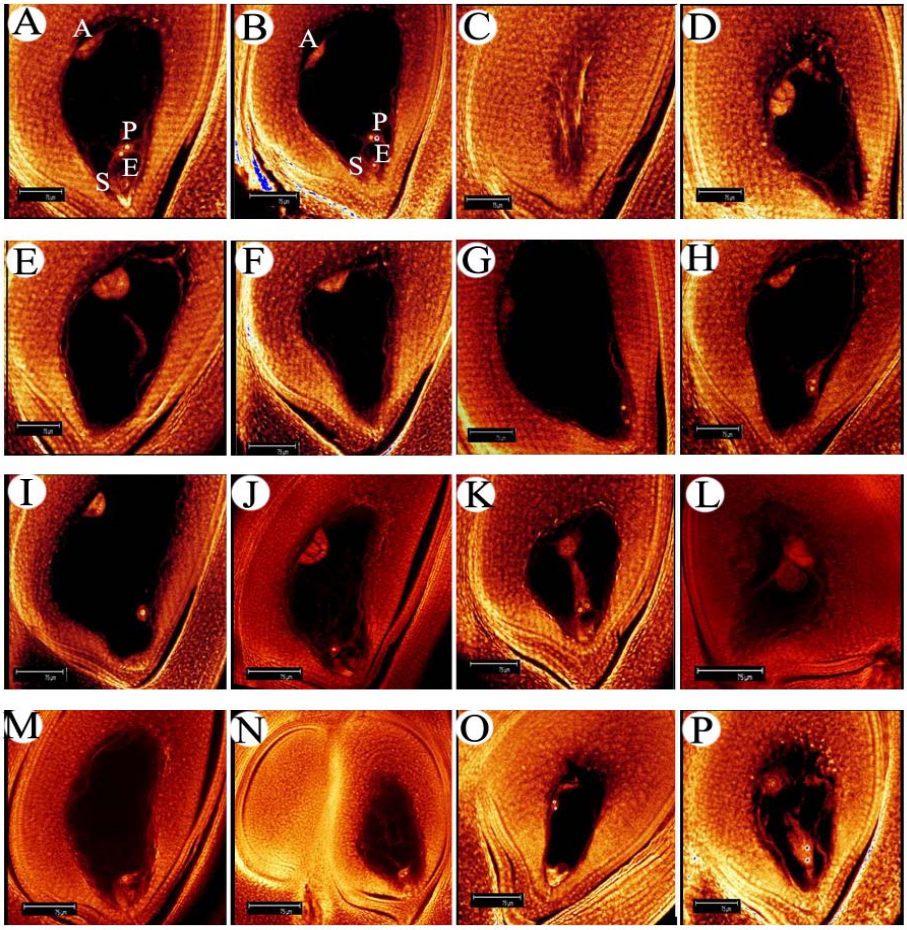

Fig. 1. Normal and abnormal embryo sacs in autotetraploid lines. Bars=75µm
A and B showing Normal embryo sac of T2 and T24 in different morphology; (C) Embryo sac degeneration in T3 ; (D) Embryo sac without female germ unit in T23; (E) Embryo sac without female germ unit in T28; (F) Embryo sac without female germ unit in T47; (G) Egg apparatus degeneration in TE; (H) Egg apparatus degeneration in T23; (I) Embryo sac with egg-apparatus degeneration and only one polar nucleus in T51; (J) Embryo sac with only one polar nucleus in T5 (K) Small embryo sac in T47; (L) Egg like structure in the middle of embryo sac near to antipodal in T2; (M) Embryo sac without antipodal in T5; (N) Two ovules in one ovary in TE; (O) Small embryo sac + Egg apparatus degeneration in TRL49 (P) Small embryo sac + Embryo sac with abnormal position of polar nuclei + Egg apparatus degeneration in TRL24.

2. Embryo sac without female germ unit (ESWF): In this embryo sac, only antipodal cells present, but female germ unit was not found. The egg cell, polar nuclei and synergids, which constitute the female germ, were degenerated (Fig. 1D-F and 2D-E). These figures illustrate the different position and size of antipodal along with ESWF.

3. Egg apparatus degeneration (EAD): Polar nuclei and antipodal cells were present but egg apparatus degenerated (Fig. 1G-I and F-I).

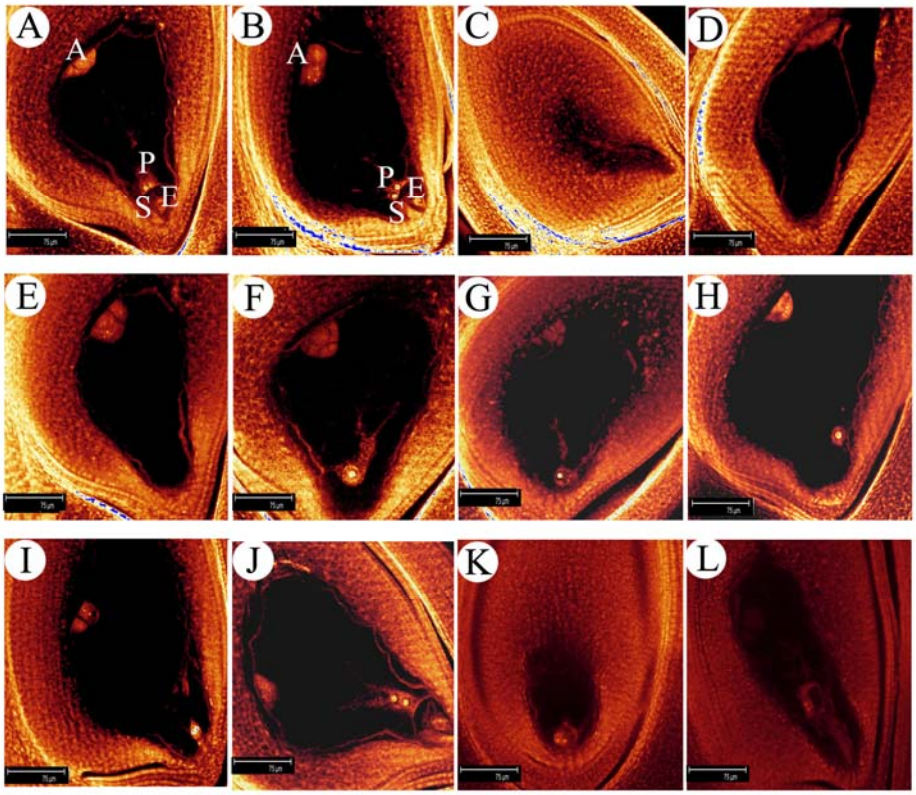

Fig. 2. Normal and abnormal embryo sacs in diploid cultivars. Bars: 75µm
A and B showing Normal embryo sac of D5 and D47, Antipodal (A), Polar Nuclei (P), Synergids (S) and Egg (E); (C) Embryo sac degeneration in D49; (D) Embryo sac without female germ unit in D3; (E) Embryo sac without female germ unit in D24; (F) Egg apparatus degeneration in D28; (G) Egg apparatus degeneration in D49; (H) Egg apparatus degeneration in D51; (I) Egg apparatus degeneration in D47; (J) Embryo sac with abnormal direction of polar nuclei in D2; (K) Small embryo sac + abnormal position of polar nuclei + no antipodal in DE; (L) Embryo sac without egg apparatus and abnormal position of polar nuclei in D5.

4. Embryo sac with abnormal number of polar nuclei (ESAP) or abnormal position (ESAN): Polar nuclei were found at abnormal position, some embryo sacs contained one polar nucleus or more than two polar nuclei and sometime no polar nucleus was found (Fig. 1J and 2J).

5. Small embryo sac (SES): The embryo sacs were smaller in size than that in normal embryo sac, which reduced to half or 1/4 to 3/4 of the normal size (Fig. 1K).

6. Other abnormal types (OAT): Some other types of abnormal embryo sacs were found during the observation, but their frequency was low. For example, embryo sac contained egg-like structure located near to antipodal cells in the chalazal part (Fig. 1L), embryo sac was lack of antipodal (Fig. 1M) and two ovules were in one ovary (Fig. 1N). Moreover, some embryo sacs were found with more than one abnormality (Fig. 1O-P and 2K-L).

Frequency of various abnormal embryo sacs in different autotetraploid lines and growing seasons: Different autotetraploid lines showed the different degree of abnormal type's embryo sacs during different seasons. Abnormal embryo sac frequencies ranged from 28.3% to 51.2% and 21.2% to 50.9% during early and later seasons in autotetraploid rice, respectively (Table 2). T test showed that frequencies of abnormal embryo sacs were significantly higher in autotetraploid rice than diploid rice. Embryo sac without female germ unit and embryo sac degeneration was more frequent types of abnormalities during early season and late season, respectively, in autotetraploid rice.

The frequency of ESD (embryo sac degeneration) was 11.2% and 11.3% during early and later season in autotetraploid lines, respectively (Table 2). The minimum and maximum number of ESD was 5.7% (Guanglu'ai 4-4x) to 18.2% (Linglun-4x) and 4.3% (Guanglu'ai 4-4x) to 19.2% (Shengnong 265-4x) during early and late season, respectively. ESWF (Embryo sac without female germ unit) consisted of 11.7% of total abnormal types in autotetraploid during both seasons, while 14.2% and 9.2% during early and late season in autotetraploid lines, respectively. ESWF varied from 8.5% to 23.2% and 5.3% to 14% in autotetraploid lines during early and late season, respectively. The mean frequency of EAD (Egg apparatus degeneration) was 5.4% in autotetraploid rice during both seasons, almost similar trend was found during both seasons. Shengnong 265-4x and IR36-4x produced the maximum and minimum number of EAD during both seasons, respectively. ESAP/ESAN explained 4.3% in autotetraploid of the total abnormal type of embryo sacs, while 4.1% and 4.5% during early and late season, respectively. Autotetraploid line "Jackson-4x" produced 6% abnormal embryo sacs during both seasons with one polar nucleus. This indicated that types of abnormal embryo sacs are more genotype dependent as compared to seasons. SES (Small embryo sac) was 2% in autotetraploid lines and almost same frequency was found during both seasons. The frequency of SES ranged from 1.0% (Jackson-4x) to 4.2% (Shengnong 265-4x) and 0.5% (Jackson-4x) to 3.1% (Linglun-4x) during early and late season, respectively. OTA (Other abnormal types) was 2.6% in autotetraploid rice, while almost similar percentage of OTA was found during both seasons. OTA ranged from 1.0% (Shengnong 15-4x) to 6.2% (Dali'nuo-4x) and 0.9% (E24-4x) to 4.5% (Dali'nuo-4x) during early and late season, respectively. There was a less difference of abnormal types of embryo sacs within genotypes during different seasons, but higher difference was found in abnormal types between genotypes during the same season, so it is concluded that seasons have less impact on aborted embryo sacs than genotypes in autotetraploid rice (Table 2). Almost similar frequency of abnormal embryo sacs were found on the base of *indica* and *japonica* types in autotetraploid lines, so subspecies have no impact on the number and types of abnormal embryo sacs.

Pollen and embryo sac fertility and their correlation with seed setting in different seasons: Temperature and rain fall data during the reproductive stage were taken from the Guangzhou meteorological station during three seasons and illustrated in Fig. 3. ANOVA was preformed to compare the maximum and minimum temperatures and rainfall during three seasons which showed highly significant difference between three seasons. The Highest maximum temperatures at reproductive phase were 36.8°C, 36.6°C and 35°C during early season 2007, early season 2008 and late season 2007, respectively. These factors might have a strong influence on the development of pollen and embryo sac.

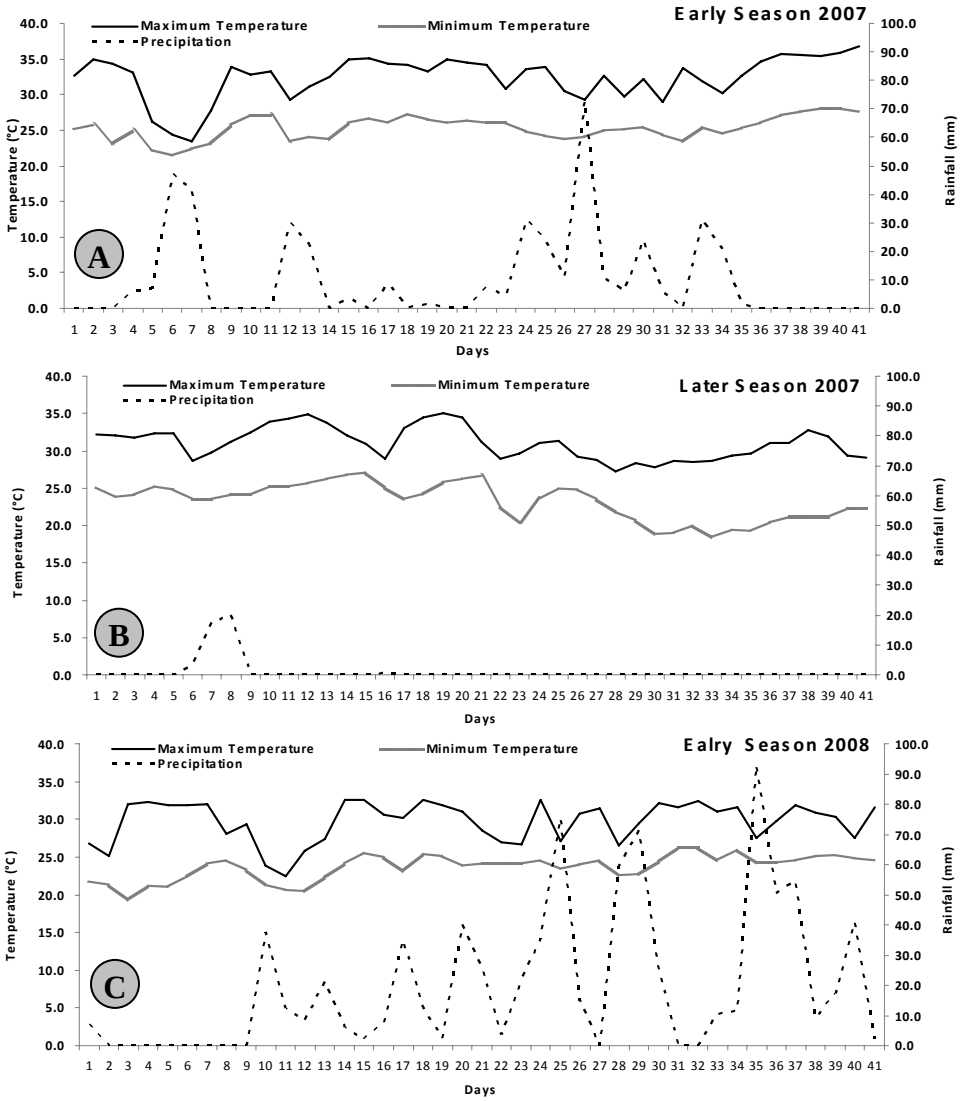

Fig. 3. Mean daily temperature and rainfall trends at reproductive stage during early season 2007 (A), later season 2007 (B) and early season 2008 (C).

Seasons effect was found significant on pollen fertility and embryo sac fertility in both diploid and autotetraploid rice, while seed setting rate of autotetraploid rice showed non-significant effect of seasons (Table 3). Diploid varieties showed normal pollen fertility, but varied under different seasons (Table 3). Higher pollen fertility (95.59%) and seed setting rate (80.89) was found during late season 2007 than other seasons in diploid rice, while autotetraploid lines also showed higher pollen fertility (77.66%) and seed setting rate (35.92%) during late season 2007. Pollen fertility and seed setting rate of autotetraploid lines was significantly lower than diploid varieties (Table 3). Non-significant correlation was found between seed setting rate and pollen fertility during

early season 2007 (Fig. 4A) and late season 2007(Fig. 4B), while a significant correlation 0.721 ($p<0.05$) was found between seed setting rate and pollen fertility during early season 2008 in diploid cultivars (Fig. 4C). Early season 2007 showed significant and positive correlation 0.715 ($p<0.05$) between pollen fertility and seed setting rate (Fig. 4D), while other seasons didn't show significant correlation in autotetraploid lines (Fig. 4E-F).

Abnormal embryo sacs found above contain no egg cell or no normal polar nuclei could not fertilize. Therefore, these embryo sacs were defined as aborted embryo sac in this paper. Seed setting rate and embryo sac fertilities were significantly higher ($p<0.01$) in diploid cultivars than autotetraploid lines (Table 3). It indicated that doubling of chromosomes increased the frequency of abnormal embryo sacs. Later season 2007 produced higher embryo sac fertility in diploid (87.4%) and autotetraploid rice (67.2%) than early season 2007 which showed lower embryo sac fertility in diploid (84.5%) and autotetraploid rice (61.7%). Embryo sac fertility was highly correlated 0.865 ($p<0.01$) with seed setting rate during early season and also found to be correlated 0.739 ($p<0.05$) during later season in diploid rice (Fig. 4A-B). In autotetraploid lines, significant correlation 0.715 ($p<0.05$) and 0.834 ($p<0.01$) was found between embryo sac fertility and seed setting rate during early and late seasons, respectively (Fig. 4D-E).

Discussion

Embryo sac is very important sex organelle for fertilization, which undergoes long developmental stages: megasporocytes, meiosis, functional megaspore, mono-nucleate embryo sac, two nucleate embryo sac, four-nucleate embryo sac, eight-nucleate embryo sac and mature embryo sac. Abnormal phenomena can occur at any development stage of embryo sac which will result in aborted embryo sac. There are four chromosomes in each homologous genome. Many abnormal behaviors of chromosome pairing were found in autotetraploid rice, which may make embryo sac abnormality in all stages from meiosis. The abnormality in mature embryo sac was the final result of these abnormal embryo sacs. So, it is important to detect the aborted mature embryo sac precisely. WE-CLSM technique makes the observation on embryo sac easier. In this paper, WE-CLSM was employed to observe ten different autotetraploid rice lines, and more than five types of abnormal embryo sac were found. Novel abnormal embryo sacs were found in different autotetraploid rice lines during different seasons. In this paper, six categories of abnormal embryo sacs were found based on their structures. Seasons have a significant effect on embryo sac fertility, but types of abnormalities were majorly controlled by genotype as compared to seasons. ESWF and ESD were the most frequent type of abnormalities during both seasons in diploid and autotetraploid rice, similar findings were reported by Hu *et al.,* (2009) in autotetraploid hybrids during early season.

We did comparative study of diploid and autotetraploid to find out the relationship of seed set with pollen and embryo sac fertility. Our results indicated that embryo sac fertility had a highly significant correlation with seed setting rate in both diploid and autotetraploid rice during both seasons while pollen fertility showed significant correlation in only one season. These results are consistent with Zeng *et al.,* (2007) but in contrast to Guo *et al.,* (2006) who found non significant co relation between embryo sac fertility and seed setting rate. Liu *et al.,* (2007) revealed that pollen fertility highly correlated with seed setting rate of autotetraploid rice. It is interesting to note that autotetraploid hybrids have higher embryo sac fertility than diploid counter parts (Hu *et al.,* 2009), but we found that autotetraploid parents have significantly lower embryo sac fertility than diploid parents.

Table 3. Pollen fertility, seed setting rate and embryo sac fertility of diploid and autotetraploid rice.

Early season 2007			Late season 2007			Early season 2008		
Pollen fertility (%)	**Seed setting rate (%)**	**Embryo sac fertility (%)**	**Pollen fertility (%)**	**Seed setting rate (%)**	**Embryo sac fertility (%)**	**Pollen fertility (%)**	**Seed setting rate (%)**
d							
93.03 ± 0.78 BC**	85.61 ± 0.55 AB**	89.1 ± 1.94 B*	98.07 ± 0.34 AB**	86.75 ± 0.46 A**	92.0 ± 0.67 AB**	93.77 ± 0.75 A**	86.41 ± 0.90 AB**
95.87 ± 0.24 AB*	87.63 ± 0.50 A**	95.3 ± 0.77 A**	98.67 ± 0.22 A**	89.57 ± 0.59 A**	94.4 ± 0.59 A**	93.80 ± 0.35 A*	88.50 ± 0.60 A**
97.77 ± 0.70 A*	77.84 ± 2.04 D**	80.7 ± 1.54 C**	97.10 ± 0.93 ABC*	77.44 ± 1.93 C*	88.7 ± 1.39 BCD*	90.37 ± 0.68 BC	70.01 ± 0.90 D*
91.87 ± 0.27 C	82.25 ± 0.45 BC**	86.7 ± 1.92 B**	95.10±0.78 BCD**	71.50 ± 2.10 D*	86.2 ± 1.35 DE**	91.90 ± 0.84 AB*	83.33 ± 0.92 BC**
93.13 ± 0.52 BC**	79.04 ± 0.73 CD**	80.9 ± 0.97 C**	93.07 ± 0.73 D*	81.09 ± 1.63 B**	82.2 ± 1.18 F**	91.53 ± 0.33 AB**	81.78 ± 1.74 C**
88.07 ± 1.36 D*	86.58 ± 2.17 A**	87.3 ± 1.02 B*	93.67 ± 0.70 D*	89.17 ± 0.15 A**	90.9 ± 0.80 ABC*	85.00 ± 1.53 D*	81.56 ± 1.40 C**
88.10 ± 2.74 D**	69.91 ± 1.11 E**	80.2 ± 1.46 C*	92.37 ± 1.51 D*	76.46 ± 0.32 C**	86.0 ± 1.02 DE*	84.67 ± 0.88 D*	61.63 ± 0.54 E*
96.20 ± 0.40 AB**	69.50 ± 1.22 E**	78.0 ± 1.76 C	97.53±0.58 ABC**	77.21 ± 0.33 C*	83.8 ± 1.94 EF**	89.33 ± 0.67 BC*	72.06 ± 1.48 D**
93.60 ± 0.61 BC*	82.03±1.61 BCD**	88.2 ± 2.50 B*	95.43±1.58 BCD**	88.56 ± 0.66 A**	88.2 ± 0.97 CD*	88.67 ± 1.45 C**	81.46 ± 0.57 C**
92.87 ± 1.44 BC*	64.13 ± 1.44 F**	78.7 ± 2.14 C*	94.87 ± 0.58 CD*	72.15 ± 0.68 D**	81.6 ± 2.12 F*	85.50 ± 0.60 D*	60.35 ± 1.40 E**
s 93.05 ± 1.01 b	**78.05 ± 2.73 ab**	**84.5 ± 0.78**^{ns}	**95.59 ± 0.69 a**	**80.89 ± 2.46 a**	**87.4 ± 0.30**	**89.45 ± 1.09 c**	**76.71 ± 3.18 b**
etraploid							
58.80 ± 1.75 D	21.97 ± 1.40 D	68.8 ± 1.76 A	61.17 ± 0.73 D	29.97 ± 1.10 E	75.0 ± 1.50 ABC	56.20 ± 2.12 E	32.13 ± 3.58 BC
73.50 ± 4.19 BC	41.48 ± 2.66 B	72.2 ± 2.17 A	81.70 ± 1.55 AB	60.11 ± 1.58 B	79.3 ± 1.30 A	72.50 ± 3.39 C	37.13 ± 2.28 B
85.87 ± 2.09 A	42.28 ± 1.99 B	66.5 ± 2.75 AB	83.67 ± 0.52 A	59.60 ± 0.49 B	72.3 ± 2.24 ABCD	84.33 ± 3.18 A	38.32 ± 3.27 AB
85.47 ± 1.85 A	54.55 ± 0.47 A	73.8 ± 1.17 A	82.53 ± 1.42 AB	37.19 ± 4.01 D	69.5 ± 1.61 BCDE	78.87 ± 1.43 AB	46.49 ± 0.36 A
76.30 ± 1.47 BC	19.22 ± 0.98 D	49.3 ± 2.60 C	77.33 ± 2.13 BC	12.47 ± 0.62 F	50.5 ± 4.04 G	76.03 ± 1.62 BC	26.79 ± 2.60 CDE
71.17 ± 3.35 C	16.42 ± 1.44 D	58.5 ± 3.91 BC	78.37 ± 1.13 ABC	17.34 ± 1.12 F	63.7 ± 3.32 DE	72.37 ± 1.53 C	27.17 ± 2.81 CD
70.10 ± 1.13 C	18.45 ± 2.12 D	53.2 ± 3.35 C	72.77 ± 3.29 C	16.63 ± 1.05 F	62.7 ± 2.89 EF	69.93 ± 0.97 C	19.75 ± 4.78 DE
80.37 ± 0.90 AB	32.88 ± 3.01 C	68.8 ± 2.52 A	84.17 ± 1.24 A	69.01 ± 1.69 A	77.2 ± 2.03 AB	74.90 ± 1.98 BC	26.60 ± 1.17 CDE
76.10 ± 2.84 BC	29.61 ± 1.24 C	53.0 ± 4.19 C	72.97 ± 1.72 C	46.28 ± 1.10 C	67.2 ± 3.61 CDE	63.00 ± 1.15 D	33.15 ± 2.28 BC
68.13 ± 2.36 C	19.48 ± 0.69 D	52.5 ± 2.93 C	81.93 ± 1.60 AB	13.09 ± 0.74 F	54.8 ± 4.34 FG	73.33 ± 2.19 BC	17.94 ± 0.87 E
s 74.58 ± 2.60 b	**30.03 ± 4.10 a**	**61.7 ± 0.34**^{**ns}	**77.66 ± 2.25 a**	**35.92 ± 6.91 a**	**67.2 ± 0.41**	**72.15 ± 2.50 c**	**30.55 ± 2.76 a**

0.05) and ** (p=<0.01) indicates that diploid cultivars are significantly different from autotetraploid lines.

l letters represent inter-varietal difference; small letters, ^{ns} and *** represent seasonal difference; * represent difference between diploid and autotetraploid. sharing similar letters are statistically non-significant.

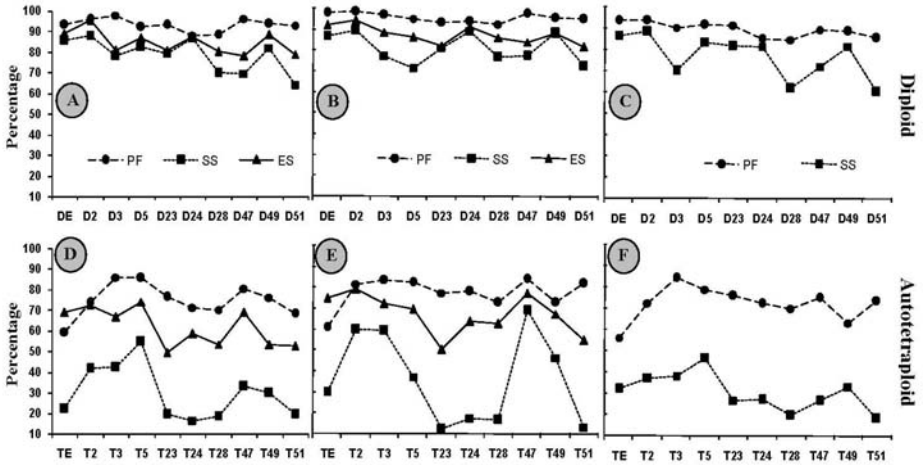

Fig. 4. Correlation analysis of seed setting rate (SS) with pollen fertility (PF) and embryo sac fertility (ES) in diploid (A, B and C) and autotetraploid (D, E and F) rice during early season 2007(A,D), late season 2007 (B,E) and early season 2008 (C,F). Ten autotetraploid lines and their diploid parents were grown at South China Agricultural University during three seasons. Each data set is a mean of three replications during each season.

Autotetraploid *Trifolium riograndense* exhibited the lesser number of fertile pollens in comparison to its diploid (Schifino & Fernandes, 1986), our study also revealed similar mechanism in diploid and autotetraploid rice. Flowering is the most crucial period in the development of rice and a few studies have been done on the effect of temperature on autotetraploid rice at this stage. Pollen development severely affected at > 33°C and further exposure to higher temperature (>35°C) during flowering period for five days caused spikelet sterility and no seed set (Satake & Yoshida, 1978). Osada *et al.,* (1973) revealed that one degree rise in temperature from 34 to 35°C increased spikelet sterility under field conditions and similar findings were reported by Horie (1993) in diploid rice under controlled conditions. We found that ≥35°C persist for one and five days at reproductive stage during later and early seasons, respectively. Embryo sac and pollen fertility reduced in early season as compared to later season which might be happened due to significantly higher temperature and rainfall during these seasons. High temperature reduced the number of germinated pollens which ultimately reduced the spikelet fertility (Matsui *et al.,* 1997, 2000, 2001; Prasad *et al.,* 2006). Seed setting rate in rice significantly reduced from exposure to high temperature during the nights (Peng *et al.,* 2004). Pollen fertility was found to be more sensitive to low temperature as compared to high temperature (Yang *et al.,* 2004). We found high embryo sac and pollen fertility at 26.5°C in contrast to 28.0°C from panicle initiation to heading. Similar findings were also reported by Zeng *et al.,* (2007) in which lower temperature produced higher embryo sac fertility. Later season also received less rainfall than early season which can also be an important factor along with other factors which affect the embryo sac and pollen fertility of autotetraploid rice.

Different genotypes showed various abnormalities and varied in frequency, similar finding were reported by Guo *et al.,* (2006). We have found that embryo sac and pollen fertility are the vital factors which affect the seed setting rate of autotetraploid rice. Further molecular studies are needed to explore the pollen and embryo sac sterility and its relation with temperature and rainfall in autotetraploid rice.

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