Genetic analysis of pollen viability: an indicator of heat stress in sunflower (*Helianthus annuus L.*)

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Abstract

Pollen viability represents sporo-gametophytic tolerance to the heat stress. Therefore, pollen fertility index under heat stress can be exploited to differentiate resistant and susceptible genotypes. Information relative to genetics of pollen viability is necessary to improve pollen viability under heat stress. Studies were initiated to develop and evaluate heat tolerant populations under controlled and field conditions. Result showed that pollen fertility index of genotypes was stable over the years. It showed moderate to high heritability, which was due to greater magnitude of genotypic effects in total phenotype. Studies showed that pollen fertility was primarily controlled by dominant type of genetic variability which showed that selection *per se* for the pollen fertility could be improved through recurrent selection. General combining analysis showed that gametophytic type of heat resistance was important in the inheritance of pollen viability. Heat resistance was dependent on the genotype of gametes as indicated from lack of relationship between mean performance of inbred line per se and their progenies for pollen viability.

Keywords: *Heat stress, Reproductive fitness, Pollen staining, Floral head, Sterility, Abiotic stress, Mobilization of reserve, Gene action, Dominance*

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Introduction

Sunflower (*Helianthus annuus*) is an important oilseed and landscape plant. Supra-optimal temperature induces significant affects over its reproductive fitness such as pollen production (Kalyar et al., 2013; Kalyar et al., 2014). The pollen viability was shown to be lost rapidly under high temperature conditions (Coast et al., 2015; Das et al., 2014; Sato et al., 2002). Pollen viability equates to its ability to live, survive and remain fertile under favourable conditions. Pollen viability, retention of pollen in the anthers and pollen germination was negatively impacted when temperature exceeds or equal to 32°C ≥ in various crop species (Coast et al., 2015; Das et al., 2014; Sato et al., 2002). The pollen abortion was noted at uninucleate stage due to abnormal development of tapetum cell when temperature was greater than 36°C resulting in yield failure in C_3 crop species (Harsant et al., 2013).

Pollen viability was determined on the basis of different staining methods, pollen germination potential on stigma or in-vitro germination of pollen grains (Rodriguez-Riano and Dafni, 2000; Satish and Ravikumar, 2010; Vaughton and Ramsey, 1991). However, pollen staining and *invitro* germination has been extensively exploited to estimate pollen viability (Patel and Mankad, 2014; Satish and Ravikumar, 2010). In- vitro pollen germination tests have been used for the assessment of pollen germination percentage and vigour over time (Chatterjee et al., 2014; Prabhushankar et al., 2005; Sulusoglu and Cavusoglu, 2014). A variety of stains have been used in the past for the assessment of pollen viability and relative estimate of fertilization potential (Abdul-Baki, 1992; Frescura et al., 2012; Gaaliche et al., 2013; Huang, 2004; Ilgin et al., 2007).

Heat stress on pollen viability relates with modification in the carbohydrate metabolism of anther development. Under favourable temperature conditions (28/22 °C), the starch accumulation in the pollen grains of tomato plant reaches maximum value 3 days before anthesis. Continuous contact of tomato plant to high temperature (32/26 °C) reduces the starch concentration and decreases soluble sugars in the pollen grains and anther walls (Pressman et al., 2002).

Impact of heat stress on pollen viability has been well documented, and subsequent discrimination of germplasm on the basis of pollen viability has been noted in various crop species (Clarke and Siddique 2004; Dane et al., 1991; Schoper et al., 1987). Data regarding genetic parameters was also needed to improve the pollen viability in species (Kalyar et al., 2014; Rauf, 2008).

In order to obtain data regarding magnitude, type of genetic variation, and heritability associated with pollen viability, experiments were carried out to determine the potential of pollen viability as selection criterion.

Materials and Methods

Experiment 1. Experiments were carried out in the experimental research area of the University College of Agriculture, University of Sargodha during the year 2009-12.

Development of sunflower population. Crosses between inbred lines were attempted during the year 2009. Parents were selected on the basis of their pollen viability under heat stress. These crosses were evlauted during the year 2010 under various temperature regimes to select promising F_1 crosses. F_2 seed was obtained by bagging the floral head of F_1 population in the field while fresh seed of the original F_1 population was obtained by attempting crosses between the parental lines through hand emasculation and pollination method.

Growth conditions for sunflower population. All the populations i.e., parents, F_1, and F_2 were grown in large plastic bags carrying 17 kilo grams of soil. Two seeds were sown in each bag, which were later transferred on thinned to single plant per bag after germination.Soil was prepared by mixing an equal amount of soil, silt and loam. Fertility and water holding capacity of the soil was raised by adding 5% of well rotten farmyard manure obtained from the landscape department of the University College of Agriculture, University of Sargodha, Pakistan.The parents and F_1 populations were represented by 15 plants while F_2 plants were represented by 60 plants in each replication. During entire crop growth cycle, plants were irrigated to field capacity (FC; 27%, w/w), which was measured through a gravimetric method (Reynolds, 1970). No visual signs of disease or insect attack were observed on plants. Fertility status of pots was raised by applying di-ammonium phosphate fertilizer (2 g pot^{-1}) after a 30-d interval. Each F_2 population was represented by 80 plants while F_1 and parents were represented by 15 plants. Plants were grown to maturity. During vegetative phase plants were kept closer to the optimum temperature 26±2; 16-h photoperiod; PPFD = 600 µmol m^{-1} s^{-1} for growth while during reproductive growth plant was subjected to the heat stress of 45.0±2.0.

Experiment II

Development of Plant material. Twelve cytoplasmic male sterile line (CMS) lines differing for heat resistance (Kalyar et al., 2013) were obtained from the department and crossed with five male lines to obtain sixty cross combinations. All CMS lines were planted in the field during the year 2013 and 2014. The plants were raised with standard production package. All CMS lines were covered with net bags and pollen from restorer lines were collected early in the morning from male restorers. The pollens were applied on floral head until stigma was completely withered.

Evaluation of experimental hybrids and parental lines. Experiments were conducted to determine pollen viability of developed sunflower germplasm. Seeds of sixty sunflower (*Helianthus annuus* L.) experimental hybrids and their parents were sown in Randomized Complete Block Design in three

replications during the year (2014 and 2015), in experimental field of the University College of Agriculture, University of Sargodha, Pakistan. All genotypes were sown at the end of March to provide heat stress during the entire reproductive phase (Fig.1). Soil had EC=2.19±0.21, PH=7.12±0.26, available K^+=231.4±26.2, available P= 17.46±5.17. The fertility of soil was raised by using inorganic urea and diammonium phosphate at the rate of 75 kg of nitrogen and 45 kg of phosphorous on acre basis.

Plant to plant distance was maintained at 20cm while row to row distance was maintained at 30cm. There were three rows of 4.5m for each genotype. Experimental field was irrigated by canal water throughout the experiment to remove deleterious effect of water stress. Fields were irrigated with canal irrigational water when moisture was lower than field capacity. Soil field capacity was visually observed. Manual hoeing was done to remove all the weeds. Regular pest scouting was done and insects were controlled through insecticide (armyworm control 200 mL lufenuron, Match ® Syngenta).

During anthesis phase, anthers were collected just prior to the dehiscence from various rows of sunflower heads at 630 hours. Pollen viability was estimated from ten plants of the middle row.

*Measurment of plant data.*100-seed mass was measured by manually counting the random sample of 100 seed and weighing on analytical balance. Three random samples were drwan from each head.

Pollen viability. Pollen viability was estimated by vital stain technique. At the time of anthesis 10 disc florets from each head row were collected at sunrise (08:00 h) from each plant. Pollen grains were squeezed from the anthers by tweezers,and pollens werecollected on clean slides. Pollen viability was tested using 2% tri-phenyl tetrazolium chloride stain. A drop of tetrazolium chloride was added to the dispersed pollen. Tetrazolium chloride reacts with H ion of anthers yielding in deep red Formazan stain, which is non-diffusible in nature.The numbers of pollen grains stained was recorded 30 min after staining under 40× Micros MCX 100 microscope (Hunnenbrun, Austria). Pollen stained in deepred Formazan color depicted as viable and fertile, while absence of deep red color indicated sterile pollen.Respective percentages were calculated. The percentage of sterile pollen was estimated as the percentage of total pollen that remianed unstained pollen.Constrastingly, pollen which took red deep stain were considered as viable.

Biometrical procedures

Analyses of variance were carried out in experiment 1 to determine the variability among the genotypes of various traits. Percentage data were transformed using excel log function before the analyses. In experiment 1, analyses were done in complete randomized design with factorial arrangements. Mean-values of the traits were compared for significance through least significance differences at $P \leq 0.05$. Data were analyzed through macros add-ins of DSAA-STAT ver. 1.01,(Onofori, 2011) using Windows Excel 2007.

In experiment 2, analysis of variance was carried out in randomized complete block design in factorial arrangement. Genetic analysis and combining ability effects were estimated according to Kempthorne (1957). Biometrical parameters such as genotypic, phenotypic, GCV% and heritability over the year were measured as outlined by Allard (1960). Heritability = (σ^2g/ σ^2p) × 100 (σ^2g = genotypic variance/σ^2 p=phenotypic variance). Genotypic coefficient of variation (GC%) = (σ^2g /X) × 100.

Results

Experiment I. Pollen viability was noted in various generations of sunflower (*Helianthus annuus* L.) subjected to heat stress after staining the pollen with tetrazolium chloride. The plant populations were exposed to the highest temperature of the day i.e. 45°C. The results have been presented in Table 1. It was shown that parental means were significantly differed from each other, and F_1 means had significant lower pollen fertility than parent with high pollen fertility. However, F_1 mean pollen fertility was lower than parent with higher pollen fertility. F_2 means of various crosses were lower than F_1 but higher than parent with lower pollen fertility (Table 1). However, F_2 population disclosed significant variation with respect to pollen fertility and plants with high pollen fertility may be selected from the population. Results showed pollen sterility tend to be dominant over the pollen fertility due to heat stress in F_1 generation. Partial dominance towards lower pollen viability was noted in the inheritance of this trait. Therefore selection should be practiced in segregating populations for high pollen fertility. Heritability for pollen sterility was moderate to high. There was strong association between plants of F_2 for pollen fertility and filled grain%. The pollen sterility increased the number of unfilled grain which reduced the 100-seed masses of genotypes under heat stress.

Experiment II. Analysis of variance of parents and their crosses was done for pollen viability which showed that there was significant ($P \leq 0.05$) variation among parents and crosses for pollen viability under heat stress (Table 2). However, variation due to contrast between parents and crosses was insignificant ($P \geq 0.05$).

Line × Tester analysis of pollen viability also showed that variation due to male and female parents was insignificant ($P \geq 0.05$) (Table 3). On the other hand, there was significant ($P \leq 0.05$) variation due to male × female interaction suggesting that dominance effects contributed to the inheritance of pollen viability. These results imply that pollen viability cannot be exploited as selection criterion for heat resistance in early segregating populations such as F_2. However, the trait could be used as dominant marker for discriminating sunflower advanced lines under heat stress.

Mean values, ranges, genotypic and phenotypic coefficient of variation have been shown in Table 4. Results showed that over all pollen viability was lower than commercial hybrid S-278 but higher than popular hybrid Hysun-33 under heat stress. Furthermore, parents and hybrids enclosed a wide range of

variability for pollen viability under heat stress. Many parents and crosses exceeded to commercial hybrids (Table 4).

Mean values of parents and their general combining ability have been shown in Fig.2. General combining ability indicated the ability of breeding line to produce superior progeny. Breeding lines with higher general combining ability values tend to have greater proportion of positive alleles affecting the trait of interest. Relationship between the mean pollen viability of parents and general combining ability indicated no relationship between these two variables (Fig 2). The results showed that it was not necessary that parents with high values for pollen viability could also produce superior progeny. However, Figure 3, indicated few breeding lines with high pollen viability and good general combining ability. For instance quadrate III was populated by breeding lines with "B-6" and "B-20" with good pollen viability and general combining ability under heat stress. Moreover, quadrate I was populated with breeding lines such as "B-2", "B-5" and "B-6" having high general combining ability with low pollen viability. Quadrate IV was occupied by breeding lines such as "B-14", "B-21", "B-3" having low combining ability and higher mean values for general combing ability. Breeding lines in quadrate I and IV may be crossed to develop transgressive segregants.

Discussion

Pollens have been good indicator of various abiotic stresses on plant and have been used to discriminate the crop germplasm against various stresses (Coast et al., 2015; Das et al., 2014; Sato et al., 2002). Different stresses reduce the photosynthates production, thus genotypes also reduce the reserve mobilization for tapetum cells which induce significant reduction in pollen development and fertility (Fu et al., 2011; Sarhadi et al., 2012). It has also shown a close association with filled grain or grain number under drought and heat stress (Kalyar et al., 2013; Nguyen et al., 2009; Nguyen et al., 2012).
Only few studies have practically improved the pollen viability in the segregating generation through selection of high pollen fertility. This is due to the lack of understanding regarding the inheritance of the traits. Information regarding the heritability of pollen viability is lacking. This study indicated substantial genetic variation among various populations of sunflower for pollen viability. Moreover, traits also showed high heritability. It has been known that high heritability in the segregating generation warranted good selection response (Kalyar et al., 2014). Moreover, magnitude and type of genetic variation associated with pollen viability was also important in improving trait and breeding procedure to be adapted for the improvement of trait (Kalyar et al., 2014). It has been known that trait having high degree of additive variance could be exploited as selection criterion in earlier segregating population and may be improved through simple selection procedures (Dudley and Moll, 1969; Verhoeven et al., 2006). Results of the study showed the importance of high magnitude of dominance effects in total genotypic variance. These results implied that recurrent selection may be practiced to improve pollen viability and to break intra-allelic interactions. However, these results were subjected to the environment

and specific population under study. In Maize and tomatoes, populations generated from diallel crosses showed preponderance of additive variance associated with pollen viability under heat stress (Dane et al., 1991; Schoper et al., 1987).

Exposure of the abiotic stress has also indirectly improved the overall fitness of plant population under stress by spontaneous selectionofheat tolerant gametes under prevailing conditions. This may be due to exposure of recessive heat susceptible alleles under haploid conditions. Clarke and Siddique (2004) discriminated tolerant pollens by exposing the F_2 parental plants to the chilling stress. It was hypothesized that under prevailing condition of chilling stress, only tolerant pollens were able to Carry out the successful fertilization. Developed seeds were used to grow single plant progenies. These progenies were selected for uniformity in segregating population to establish advanced breeding lines. The developed advanced lines were shown to have better resistance against the chilling stress. Similarly, Kalyar *et al.* (2013) indicated that population fitness was increased after exposing the segregating population to the natural heat stress cycle. It was noted that heat stress could only allow the heat resistant pollen to germinate over the stigma resulting in the evolution of heat resistant plant types.

General combining ability (GCA) of parental lines was estimated on the basis of their ability to produce superior progeny. GCA and mean value of the parents *per se* showed thatsome parental lines with high pollen viability may show poor GCA values. This showed that it was not necessary for high valued parental line to also produce a progeny of high pollen viability. This may be due to the presence of greater proportion of negative alleles within parents reducing the overall performance of its progenies (Hallauer *et al.,* 2010). General combining analysis showed that heat resistance was controlled through genotype of gametes. Gametophytic type of heat resistance was important in the inheritance of pollen viability i.e. heat resistance was dependent over the genotype of gametes since there was no relationship between the performance of inbred line *per se* and their progenies. However, few parental lines were identified with high mean and GCA value. These parents could be favoured for the development of heat resistant hybrids.

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