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Male sterility induced by chemical SQ-1, as an effective male specific gametocide in maize (*Zea mays*)

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Abstract

Induction of male sterility by male gametocides holds great potential in utilization of heterosis aside from nuclear encoded male sterility (NMS) and cytoplasmic male sterility (CMS) applied in crop breeding. In the present study, we reported a recently synthesized gametocide SQ-1, a pyridazinone derivative used for induction of male sterility in maize. A reference dosage of chemical SQ-1 at 5.0 kg ha^{-1} was sprayed in five genetically unrelated maize cultivars at the plant developmental stage L8-L10 (8 to 10 leaves developed after the above-ground seedlings), and the results showed that the SQ-1 treated plants exhibited an intact female organ that remained vigorous to alien pollination, and > 90% of male sterility reached by assaying pollen viability albeit fewer seeds were residually obtained. Besides, the present data also demonstrated that the SQ-1 induced male sterility was not severely affected by different maize genotypes. Therefore, as an alternative of NMS and CMS, the gametocide SQ-1 could be a substitution approach in maize breeding program considering its rapidity and flexibility.

Keywords: male sterility, gametocide, SQ-1, hybrid vigor, maize

Introduction

Male sterility is defined as a failure of plants to produce functional anthers, pollens and/or male gametes during their reproductive stages. Male sterility was firstly observed as abortion of anthers within species and interspecies crosses in Nicotiana (Rieseberg, 1997). Subsequently male sterility was exploited to utilize heterosis in plant breeding. Theoretically, various approaches could be used for harnessing heterosis by promotion of cross-pollination such as removal of anthers or male flowers, use of cytoplasmic or nuclear-encoded male sterility, application of male-specific gametocides and so on.

The basic advantages of gametocide application allow use of any parental combinations for production of hybrids. Furthermore, in comparison with CMS system, an effective gametocide could be used for production of a higher number of parental combinations and allows for evaluation of their combining capacity and genetic values. This approach substantially reduced the time required for development of CMS system. But as an optimal alternative, the good gametocides should be safe and have low phytotoxic effects allowing better quality seeds, and can be widely used in a great number of genotypes (Cross and Ladyman, 1991).

Maize is confirmed to show a great vigor of hybrid by outcross, and three major types of CMS were already identified and used in maize: CMS-T (Texas), CMS-S (USDA) and CMS-C (Charrua). CMS-T was extensively used in the year 1950's and 1960's, but showed extremely susceptible to *Bipolaris maydis*, pathogen fungi that generated a disease called Southern corn leaf blight (Levings, 1990). So far, commercial production of maize hybrids stands upon utilization of cytoplasmic C and S types (Sofi et al, 2007). Nevertheless, the susceptibility of fertility restoration remains environmentally a problem faced by plant breeders. Optionally, the use of gametocides to create super combinations in maize could be a good alternative to improve maize yields.

SQ-1 was a newly synthesized and registered gametocide that was tested to be environmentally friendly, and SQ-1 specifically hindered the development of mature pollens at the postmeiotic stage (Cheng et al, 2004). SQ-1 was used for production of wheat hybrids (Wang et al, 2005; Li et al, 2007) and millet (Cui, 2008; Song et al, 2011). > 95% of wheat and millet plants became specifically male sterile after application of SQ-1 at a final concentration of 5.0 kg ha^{-1}, but the low-effective cross-pollination in these selfing plants (wheat and millet) caused a very lower seed-set that greatly eclipsed the potential application of SQ-1 in selfing plants.

In the present study we attempted to test the effectiveness of the gametocide SQ-1 in inducing male sterility in maize. By examining pollen viability, our results firstly showed that the SQ-1 could efficiently induce male sterility in maize when applied with a reference dosage of 5 kg ha^{-1} at the plant growth stage L8-L10 (8 to10 leaves formed after the above-ground sprouting) although residual shrunken seeds were

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obtained by cross-pollination with alien female inflorescences. Furthermore, the SQ-1 induced male sterility was not severely affected by the different maize genotypes. In general, the present results identified a potential use of gametocide SQ-1 for inducing male sterility to exploit hybrid vigor in maize breeding program.

Materials and Methods

Plant material and growth conditions

In the study, the most widely grown maize cultivars, kindly provided by the Agriculture Institute of Henan (Zhengzhou, Henan) were used to assay effectiveness of the gametocide SQ-1, including Jixiang-1, Zhengdan-958, Yuanyu-20, Xundan-22 and Denong-20, and no genetic relatedness was identified according to their parental pedigrees. All the seed-derived plants were grown in the designed experimental field of Zhengzhou Univeristy (Zhengzhou, Henan) with the average annual temperature and rainfall of 14.3°C and 640 mm respectively. Three independent biological replicates for each cultivar were randomly designed and plotted, and at least 150 single plants in each replicate were treated with clean water (as controls) and chemical SQ-1. Besides, compound fertilizer and irrigation were applied according to the plant growth condition.

Spraying of SQ-1 at a reference dosage at the specific growth stage

The gametocide SQ-1 was kindly provided by the Key Laboratory of Crop Heterosis of Shaanxi Province (Northwest A&F University, Yangling, Shaanxi). The growth of maize plants was staged according to the number of leaf formed after planting, for example, 33 to 35 days after planting at the 6-leaf growth stage (L6), 54 days after planting at the 10-leaf growth stage (L10) (Raun et al, 2005). A reference dosage of 5 kg ha^{-1} of SQ-1 was sprayed at a period of about 35-40 days after planting. At this period (L8-L10), the plants usually exhibit great variability and vulnerability that was easily affected by environmental stresses (Raun et al, 2005), and the meristem was determined to form a tassel when all vegetative nodes were initiated at 8-leaf growth stage (Irish and Nelson, 1991). Cytologically, microsporogenesis in the developing tassel (about 5 - 6 cm in length) was usually observed at this stage. As a control, an equal amount of clear water was sprayed (water treatment, WT) in comparison with plants treated with chemical SQ-1.

Viability assay of mature pollen grains by three different methods

At anthesis, the fresh mature pollens were collected from each treated maize cultivar, and the viability of pollens was determined by three methods: 1) staining with I_2-KI solution (I_2-KI staining), prepared with 2% wt/vol iodine, 5% wt/vol potassium iodine and 20% wt/vol chloral hydrate; 2) triphenyl tetrazolium chloride staining (TTC staining) for testing the presence of dehydrogenase in the viable pollens. The solution consisted of a 1% of the substrate 2, 3, 5-triphenyl tetrazolium chloride (TTC), and the pollen grains were considered viable if they turned deep pink (or dark red) after 2-3 h incubation at 37°C; and 3) *in vitro* germination. The media composition was optimized by testing a range of reagents used in the piloting experiment, and the germination of pollen grains was evaluated on optimized germination medium (0.8% agar, 1.0 mol sucrose, 1.58 mmol boric acid, and 1.0 mmol calcium nitrate) in petri dishes after incubation overnight (or at least 6 h) at 37°C for full growth of pollen tubes. The stained (germinated) pollens were observed with light microscope (Olympus, Japan), and the quantified value was the mean of results from three independent experiments. In vitro germination, a pollen grain with a growing pollen tube was considered germinated. The pollen germination rate for each line was calculated using three independent biological replicates, and each observation involved examination of at least 500 pollen grains to assess pollen viability.

Evaluating residual fertility of pollen grains from SQ-1 treated plants

Mature pollens from both WT and SQ-1 treated plants were collected to evaluate residual viability by hand-pollination with alien female organs. Meantime, the female inflorescences of the SQ-1 treated plants were manually crosspollinated with fresh alien pollens to assay reproduction potential of female organs after SQ-1 treatment.

Results

Morphological development of maize cultivars was not severely affected by SQ-1 treatment

SQ-1 was a newly synthesized pyridazinone derivative (Supplementary Figure 1), as a registered plant

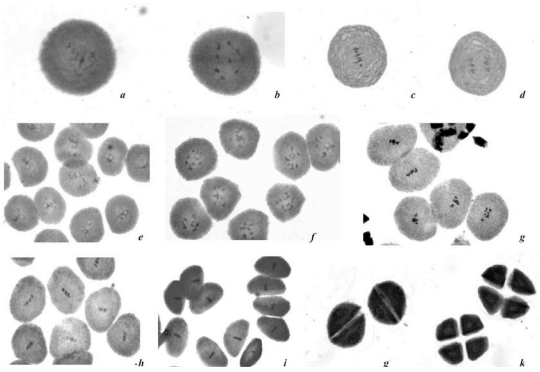

Figure 1 - Normal male meiosis observed of the SQ-1 treated plant. During meiosis I, chromosomes gradually condense at prophase I (a, b, e, f); pairing of homologous chromosomes at metaphase I (c, g, h); separation of homologous chromosomes at anaphase I (d); dyad formation during late telophase I (g); during meiosis II, chromosomes align in middle metaphase II (i); tetrad formation during late telophase II (k).

growth regulator for efficient use in the production of cereal hybrids including wheat (*Triticum aestivum*) (Cheng et al, 2004; Liu et al, 2007), proso millet (*Panicum miliaceum*) (Cui, 2008), and foxtail millet (*Setaria italica*) (Song et al, 2011). The chemicals of this sort usually suppress post-meiotic microspore development into normal functional pollens (Figure 1), allowing for cross-pollination by adjacent untreated plants (Cross and Ladyman, 1991). With a reference dosage of 5.0 kg ha^{-1} applied, when the vegetative nodes were completely initiated (stages L8-L10), the morphological development of male and female inflorescences (tassels and ears) was then investigated (Figure 2), and the results showed that the morphological formation of male and female inflorescences was not greatly affected by the applied dosage, in contrast with plants treated with water, but the SQ-1 treated plants generally exhibited a slightly reduced height at the heading stage. Besides, the pilot experiment showed that earlier application of chemical SQ-1 affected normal development of tassel internodes, but the development of female inflorescence was intact. This observation indicated that SQ-1 could be a potentially specific inhibitor on the development of male inflorescence in maize.

Pollen sterility was specifically induced by SQ-1 treatment

The viability of mature pollen grains from SQ-1 and water-treated (WT, control) plants was evaluated by different methods including I_2-KI stain, TTC stain and germination *in vitro* (Figure 3). For the I_2-KI staining, the fertile pollen grains were deeply stained stuffed with starch ingredient (Figure 3A), but the sterile pollens usually maintain stainless with less starch content (Figure 3B). Comparatively, when assayed by TTC method, the fertile pollen grains (from WT, control) were stained in deep pink with distinct round nucleus (Figure 3C) owing to the presence of dehydrogenase, but sterile pollens were lightly stained or even achromatous (Figure 3D) from SQ-1 treated plants. Besides, in vitro germination of pollen grains was performed from both WT and SQ-1 treated plants, the assaying precisely showed that fertile pollens of the control plants developed an extended tube after incubation in 37°C (Figure 3E), but the sterile pollens of SQ-1 treated plants did not produce lengthy growth tube at all (Figure 3F).

The statistical analysis was conducted to quantify the aborted and fertile pollens of treated plants as well as controls. These different methods produced a slightly varying percentage of pollen viability respectively (Supplementary Figure 2). However, regardless of the varying percentage of sterile pollens, the present results showed that SQ-1 could effectively induce male sterility in maize.

Furthermore, to test the pollen viability *in planta*, the pollens of SQ-1 treated plants were collected and manually crosspollinated to alien female inflorescences of the untreated plants, and the seed-set was determined in the several weeks (Figure 4). The results showed that only a few of shrunken seeds were detected. The reproductive potential of female inflorescences of treated plants was tested by cross pollination with alien pollens, and the fully-developed seeds were produced after 2-3 week post-fertilization development.

Genotype difference did not affect male sterility induced by chemical SQ-1

To analyze if male sterility induced by SQ-1 had a characteristic of genotype specificity, the genetic relation of different maize cultivars was firstly identified according to their parental pedigree, and then male sterility was statistically analyzed among different maize cultivars. The result showed that the used maize cultivars were highly induced male sterile by SQ-1, and no difference of significance (P=0.05) was statistically detected, though a significant differ-

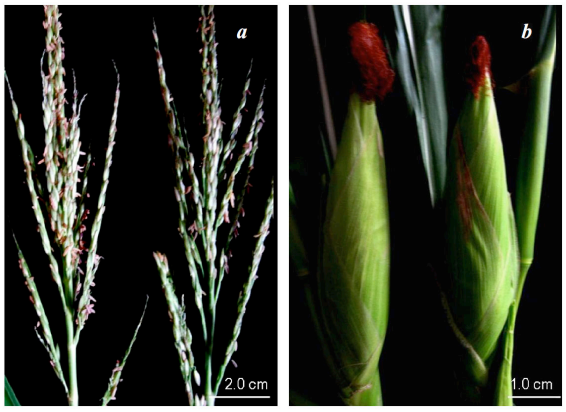

Figure 2 - Morphological features of tassels (a) and ears (b) in maize cultivar Zhengdan-958 treated with water (control, left) and SQ-1(right). No obvious difference was observed between control and SQ-1 treated plants, and both male tassels and female ears were normally developed.

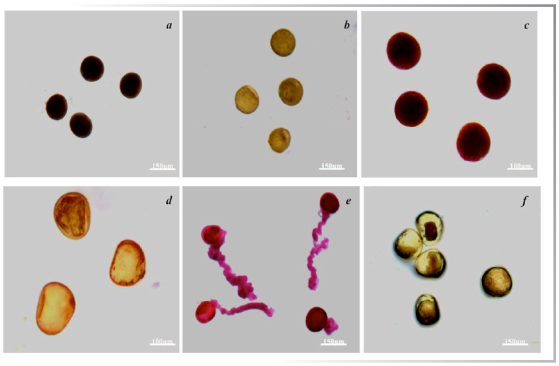

Figure 3 - Microscopic observations of pollen viability (Zhengdan-958). Fertile (a) and sterile pollen (b) identified by I_2-KI stain from WT (as a control) and SQ-1 treated plants respectively; the TTC stain showed fertile pollen (c) from WT plant and sterile (d) from SQ-1 treated plants; and germination assaying showed the fertile (e) and sterile (f) pollen grains collected from control and SQ-1 treated plants, respectively.

Figure 4 - The seed-set of SQ-1 treated plants. a) The well developed seeds of the SQ-1 treated plant crosspollinated with normal pollens; b) The undeveloped female inflorescence of the SQ-1 treated plant without pollination; c) Several developed seeds of wild-type plant crossed with the pollens collected from SQ-1 treated plant; d) The developed seeds of wild-type plant after pollination with normal pollens (as a control).

ence existed among three different assaying methods (Supplementary Figure 3). As expected, due to high starch contents in cereal plants, the I_2-KI staining method produced a higher proportion of pollens stainable as falsely male fertile in both control and SQ-1 treated plants, but for TTC staining, pollen fertility from treated plants showed an intermediate between I_2-KI stain and germination *in vitro*. Nevertheless, the present data indicated that SQ-1 enabled induce pollen abortion without preferred genotypes in maize.

Discussion

Male sterility could be induced through different approaches (Supplementary Figure 4). For instance, cytoplasmic male sterility (CMS), a maternally inherited trait was caused by incompatibility of nuclear and cytoplasmic genomes (Ruiz and Daniell, 2005). Three major types of cytoplasm, namely C (Charrua), T (Texas) and S (USDA) were used in maize, which were classified according to differential sterility expression in response to restorer (*Rf*) genes (Zabala et al, 1997), mitochondrial DNA restriction digest patterns (Pring and Levings, 1978), and compliments of low MW plasmids (Kemble and Bedrock, 1980). Although this genetic male sterility provides a suitable means for the production of hybrids, there are problems with this approach, the production of the maternal lines usually requires time-consuming crosses to incorporate the male-sterility genes. The effective and reliable fertility restoration genes must also be available in an agronomically acceptable form. Furthermore, incomplete male sterility or breakdown of the restoration through genetic reversion or environmental sensitivity cannot be tolerated in a commercial program.

As an alternative, the gametocides could also provide an alternate, workable system for inducing male sterility, which was rapid, flexible and would not require fertility restoration (Rowell and Miller, 1971). The major advantage of using gametocides was that almost any compatible parents may be used as a male or female to test potential of vigor (Kofoid, 1991). Besides, gametocides facilitate cross breeding in plant species with perfect flowers by selectively sterilizing male inflorescence or by interrupting microsporogenesis and promote cross pollination and thus offers opportunities to develop hybrids (Collantes et al, 1999). For example, two chemicals were used in France to produce wheat hybrids: Genesis (Monsanto) and Croisor (Hybrinova) (Blouet et al, 1999). In developing countries, such as China and India, a number of chemicals were investigated for use as gametocides for production of hybrid crops such as wheat (Adugna et al, 2004; Li et al, 2007), rapeseed (Yu et al, 2006), millet (Cui, et al, 2008; Song et al, 2011), and great progresses were made to improve crop yield.

SQ-1 was a recently synthesized gametocide, and used for selectively sterilizing male inflorescence in wheat (Cheng et al, 2004; Wang et al, 2005; Liu et al, 2007), millet (Cui, 2008; Song et al, 2011). In the present study, the data demonstrated that the chemical SQ-1 could also selectively hinder the post-meiotic development of functional pollens with perfect female inflorescence and preferentially induce male sterility in maize, which indicated SQ-1 could be a promising gametocide in facilitating selection of super combinations in maize breeding except for genetic approaches.

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Figure 1 The molecular structure of SQ-1. SQ-1 is a pyridazinone derivative, and the letters X, Y, Z represent the distinct functional groups respectively.

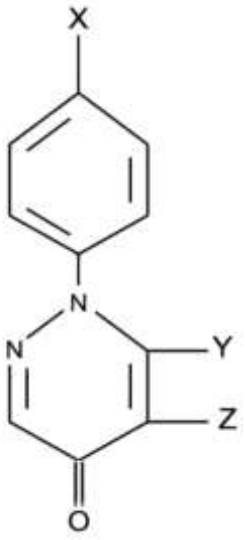

Figure 2 The analysis of relative pollen viability from the five Maize cultivars

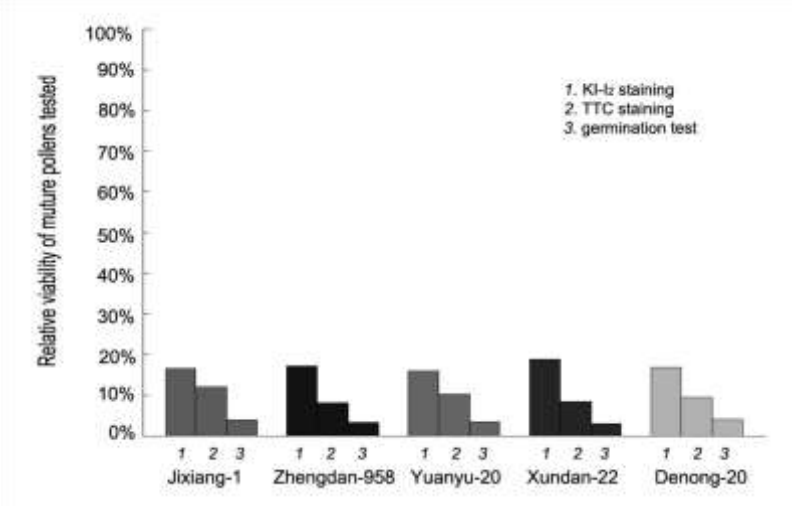

Figure 3 Comparative analysis of different methods for assaying of pollen viability. "*" means a significant difference exists at the level P=0.05

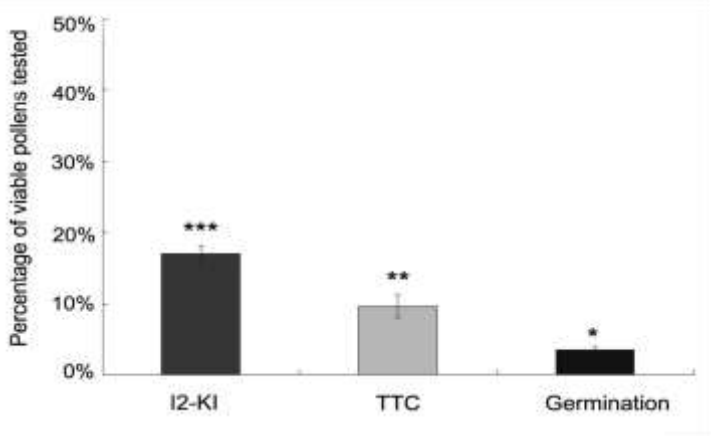

Figure 4 Genetic male sterility and gametocide-induced male sterility. Nuclear male sterility (*a*), characteristic of Mendelian inheritance, due to nuclear mutation leading to recessive alleles for male sterility, which could be further screened for pure male sterility; cytoplasmic male sterility (*b*), a non-Mendelian inheritance, but a typical interaction between nuclear and cytoplasmic genes, the male sterility could be induced by specific combination of nuclear gene (recessive, green) and mitochrondrial factors (red); chemical-induced male sterility (*c*), non-heritable, causing disruption of normal development of pollens after properly applied at a specific stage of plant growth, and could be used for selecting combinations of potential vigor in plant breeding program.