

Influence of Air Temperature on the Stability of Cytoplasmic Male Sterility (CMS) in Maize (*Zea mays* L.)

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Cytoplasmic male sterility (CMS) is a maternally inherited trait that suppresses the production of viable pollen. CMS is a useful biological tool for containment strategies to reduce or prevent gene flow and cross-pollination to facilitate coexistence of genetically modified (GM) and non GM-crops in cases where it is required. CMS is reversible and can restore to fertility in the presence of nuclear restorer genes (*Rf*-genes) and by environmental impacts. The aim of this study was to investigate the influence of air temperature on the stability of CMS maize hybrids under defined greenhouse conditions. Three CMS maize hybrids were grown in three different temperature regimes. Tassel characteristics, pollen production, and fertility were studied. The CMS stability was high in hot air temperatures and decreased in lower temperatures. The extent of these phenomena was dependent on the CMS maize genotype and should be known before using CMS for coexistence purposes.

Key words: air temperature, biological containment, coexistence, cytoplasmic male sterility (CMS), genetically modified (GM) maize (*Zea mays* L.), restoration of fertility.

Introduction

In certain cases, the cultivation of genetically modified (GM) maize might require a high level of containment/confinement to ensure coexistence with agricultural production methods that do not cultivate GM plants, such as organic farming. A single maize plant develops a huge amount of pollen (Uribelarrea et al., 2002; Westgate, Lizaso, & Batchelor, 2003), but only 5% of this amount pollinates its own silk (Poehlmann & Sleper, 1995). Reliable containment/confinement methods are necessary to avoid or reduce cross-pollination of the maize pollen into neighboring fields. Therefore, several containment methods such as distance, border rows, etc., are already applicable. In countries with a longer sowing season, dispersed sowing dates can be selected, as is common in Spain (Brookes et al., 2004; Messeguer et al., 2006).

One reliable biological confinement method is the use of cytoplasmic male-sterile maize plants (Bückmann, Hüsken, & Schiemann, 2013). Cytoplasmic male sterility (CMS) is a maternally inherited trait that prevents the development of functional pollen (Laser & Lersten, 1972; Schnable & Wise, 1998). It results from a loss-of-function mutation in the mitochondrial genome (Chase & Gabay-Laughnan, 2004) that causes a dysfunction of the respiratory metabolism and an abnormal production of male gametes (Budar, Touzet, & De Paepe, 2003; Chase, 2006). Thus, plants develop little or no vital pollen, but the female fertility is not affected.

Three main types of CMS are known within maize (Sofi, Rather, & Wani, 2007): CMS-T, or Texas cytoplasm (Levings, 1993; Rogers & Edwardson, 1952); CMS-S, or USDA cytoplasm (Jones, Stinson, & Khoo, 1957); and CMS-C, or Charrua cytoplasm (Beckett, 1971). Their differences are based on different restorers of fertility genes (*Rf*). Even though the determination of CMS occurs extranuclear, these nuclear *Rf* genes can compensate for the CMS effect of the cytoplasm (Schnable & Wise, 1998). As a result, tassels can be partly restored or become even fertile with more or less vital pollen in the first-generation progeny (F₁). Apart from the internal interactions between mitochondrial and nuclear genes, fertility of many CMS plant species can also be restored by environmental impacts such as heavy rain, extreme heat, etc. (Fan & Stefansson, 1986; Marshall, Thomson, Nicholls, & Patrick, 1974; Peterson & Foskett, 1953; Sarvella, 1966; Weider et al., 2009). The involved mechanisms are still unclear.

CMS in maize was tested successfully as a reliable confinement method to reduce cross-pollination. In a two-year study, Bückmann et al. (2013) cultivated different CMS maize hybrids in big plots (roughly 3,300 m²) in three different environments in Germany and could verify that the level of cross-pollination was drastically reduced compared to a conventional and fully fertile maize hybrid. Up to 99% lower cross-pollination rates were measured. These studies also led to the conclusion that the climate had an impact on the level of

Table 1. Temperature regimes of the greenhouse experiments.

Temperature regime	Setting	Night / day
Cold	Sowing	21°C / 25°C
	After 14 days	18°C / 25°C
	After 21 days	16°C / 21°C
Warm	Sowing	10°C / 15°C
	After 14 days	15°C / 20°C
	After 21 days	18°C / 25°C
Hot	Sowing	15°C / 20°C
	After 14 days	15°C / 25°C
	After 21 days	18°C / 28°C
	After 35 days	22°C / 35°C

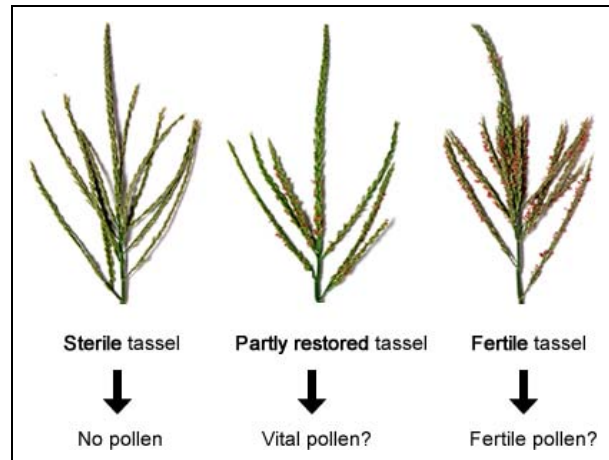
sterility, which, itself, was based on the genotype. One trial year was consistently colder than the long-standing mean except the first half of July, when temperatures increased strongly at all locations (Bückmann et al., 2013). The CMS maize hybrids expressed a higher level of sterility compared to the second trial year. Interestingly, several later-developed plants (delay of about 10 days) were detected that arose from a delayed crop development due to cold and wet weather conditions earlier in the season. These plants bloomed in roughly 2°C lower temperatures and developed fertile tassels with a large amount of pollen, while most of the earlier flowers of the same hybrid were sterile. These observations raised the question of whether the air temperature might have an impact on the CMS trait.

The aim of this study was to investigate the influence of the air temperature on the CMS stability of maize hybrids and—if produced—the vitality of pollen under defined greenhouse conditions. The results will provide more detailed information about the applicability of CMS maize hybrids under different environmental conditions as a tool for coexistence purposes.

Material and Methods

Two experiments were carried out to test the influence of air temperature on the level of CMS sterility. The first experiment was a pre-test with two CMS maize hybrids to gather a first impression of a possible temperature impact on the tassel development. After a positive testing, the second trial was carried out in the same way but with three CMS maize hybrids and an additional test of vitality of the pollen.

Three different air temperature regimes (cold, warm, hot) were used, each in separate greenhouse chambers (Table 1). The cold regime started with temperatures of

**Figure 1. Tassel characteristics of CMS maize plants.**

21°C at night and 25°C during the day provided positive initial conditions. The temperatures were reduced stepwise to 16°C at night and 21°C during the day. The warm regime was conducted as a situation following the average outdoor temperatures in Germany. Seed sowing and germination were performed at 10°C at night and 15°C during the day, respectively. From three weeks after sowing until harvest, the plants were grown at 18°C at night and 25°C during the day. Under the hot temperature regime conditions, sowing was performed at 15°C and 25°C, respectively, and temperatures were increased stepwise to reach 22°C at night and 35°C during the day after 35 days.

In daytime, the plants were illuminated 16 hours by 5,000 Lux. Eight plants per CMS hybrid and temperature regime were cultivated in 30l plastic pots filled with standard greenhouse soil based on crumble peat. Irrigation, fertilization, and plant protection were performed in accordance with the greenhouse standard of the Julius Kühn-Institute (JKI). The CMS maize hybrids tested in the greenhouse were also used in field trials to test their potential as a biological confinement tool (Bückmann et al., 2013).

In the first experiment, the CMS hybrids Torres (S-cytoplasm, Kleinwanzlebener Saat-zucht AG, Germany) and DSP2 (T-cytoplasm, Delley Seeds and Plants Company, DSP, Switzerland) were grown. In the second experiment, the CMS maize hybrid Zidane (S-cytoplasm, Kleinwanzlebener Saat-zucht AG, Germany) was added. In both experiments, the tassels were assessed whether they stayed sterile, were partly restored, or even fertile (Figure 1). Vitality tests using self-pollinations were done with all plants in the second experiment. Developed kernels were counted afterwards.

Table 2. Tassel characteristics of CMS maize hybrids in different temperature regimes (two experiments).

CMS hybrid	Temperature regime	1 st experiment		2 nd experiment	
		Tassel	Pollen	Tassel	Pollen
DSP2	Hot	100% sterile	100% no	100% sterile	100% no
		0% partly restored	0% few	0% partly restored	0% few
		0% fertile	0% many	0% fertile	0% many
	Warm	0% sterile	0% no	0% sterile	0% no
		50% partly restored	0% few	11% partly restored	6% few
		50% fertile	100% many	89% fertile	94% many
	Cold	0% sterile	0% no	0% sterile	0% no
		0% partly restored	0% few	0% partly restored	0% few
		100% fertile	100% many	100% fertile	100% many
Torres	Hot	100% sterile	100% no	100% sterile	100% no
		0% partly restored	0% few	0% partly restored	0% few
		0% fertile	0% many	0% fertile	0% many
	Warm	0% sterile	0% no	0% sterile	0% no
		100% partly restored	100% few*	100% partly restored	100% few*
		0% fertile	0% many	0% fertile	0% many
	Cold	0% sterile	0% no	0% sterile	0% no
		100% partly restored	100% few	100% partly restored	100% few
		0% fertile	0% many	0% fertile	0% many
Zidane	Hot			0% sterile	100% no
				100% partly restored	0% few
				0% fertile	0% many
	Warm			0% sterile	0% no
				54% partly restored	97% few
				46% fertile	3% many
	Cold			0% sterile	0% no
				60% partly restored	70% few
				40% fertile	30% many

* very weak

The results of the flower assessments are expressed as a percent of the total [%]. The kernels counted per ear are calculated by two-way-ANOVA (Origin software, version 8.1).

Results

In both experiments, Torres was the CMS hybrid with the highest level of sterility (Table 2). It stayed completely sterile under hot temperature conditions (22°C/35°C). In warm and cold temperatures, all Torres plants developed partly restored tassels and a reduced amount of anthers. These anthers produced a very small amount of pollen, especially at 18°C/25°C.

DSP2 stayed 100% sterile under hot temperature conditions. Under decreasing temperatures, DSP2 developed partly restored and—mainly—fertile tassels

with a large amount of pollen. Under a cold environment, all plants were fertile.

Unlike the results seen in Torres and DSP2, hot temperature conditions (22°C/35°C) resulted in partly restored tassels in Zidane, but no pollen was produced. Colder temperatures resulted in a higher number of fertile tassels, while the amount of pollen produced was lower compared to DSP2.

Kernels were developed in accordance with the tassel development (Figure 2). No CMS maize hybrid developed kernels under the hot temperature regime because no pollen was produced. Torres did not produce kernels under warm conditions either, though it produced a very small amount of pollen. Just one plant developed two kernels on one ear and, therefore, a standard deviation (SD) of 1 was calculated (Table 3). How-

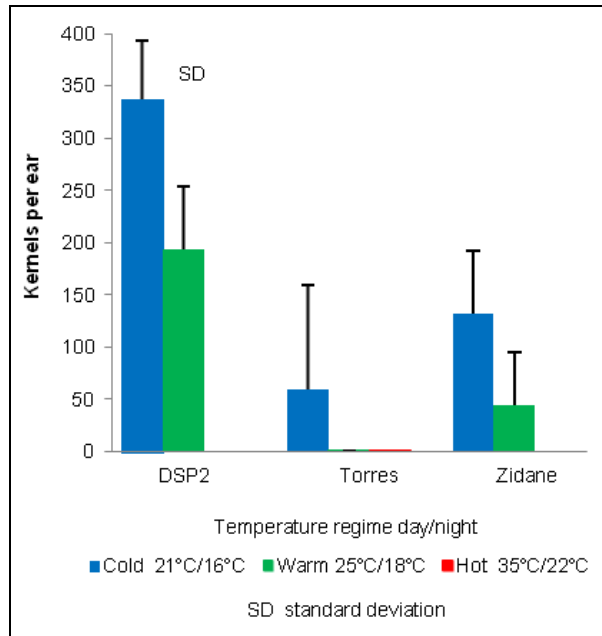


Figure 2. Kernels per ear after self-pollination of three CMS maize hybrids in different air temperature regimes.

ever, under the cold temperature regime, Torres produced 60 kernels per plant on average. The SD of 100 (Table 3, Figure 2) indicates the wide array of results. Two plants developed more than 150 kernels, while others did not produce one.

DSP2 and Zidane developed many more kernels when temperatures decreased. Under warm temperature conditions, DSP2 produced an average of 193 kernels, while Zidane developed 44 kernels on average, but the spread of kernels developed per plant was high (SD 110). Low temperatures of 16°C/21°C increased the number of kernels per ear for DSP2 and Zidane up to mean values of 337 and 132, respectively (Figure 2, Table 3).

The results were statistically significant at a level of $p=0.05$ (Table 4). All three temperature regimes differed significantly. The temperature influence on DSP2 was significantly different from that on Torres and Zidane. These two hybrids did not differ from each other on a statistical basis.

In summary, the level of sterility of the CMS maize hybrids tested was dependent on the temperature regime, and the extent of the loss of sterility was dependent on the genotype.

Table 3. Kernels per ear after self-pollination and standard deviations of three CMS maize hybrids in different temperature regimes.

Temperature regime	DSP2	Torres	Zidane
Cold (21°C / 16°C)	337	60	132
Standard deviation	56	100	60
Warm (25°C / 18°C)	193	0	44
Standard deviation	61	1	110
Hot (35°C / 22°C)	0	0	0
Standard deviation	0	0	0

Table 4. Significance of temperature and genotype influences on the amount of kernels produced, 2-way anova, $p = 0.05$.

Significance level $p=0.05$	DSP2	Torres	Zidane
Temperature regime	a	b	b
	Cold 21°C/16°C	Warm 25°C/18°C	Hot 35°C/22°C
CMS maize hybrid	a	b	c

Different letters = significant differences ($p = 0.05$)

Discussion

The objective of this study was to verify whether air temperature can affect the stability of the CMS trait of maize hybrids. The results will help to understand the climatic impacts on fertility restoration and to better define conditions for the applicability of CMS maize hybrids for coexistence purposes. In this study, three different CMS maize hybrids were cultivated under three different air temperature regimes using greenhouse conditions.

Hot air temperatures (22°C/35°C) did not affect the sterility of the CMS maize hybrids tested. DSP2 and Torres stayed completely sterile, while Zidane developed partly restored tassels but no pollen. With decreasing temperatures, the level of restoration to fertility increased. Hence, the results show clearly the influence of air temperature on the CMS trait, which might be explained by the sensitivity of *Rf* gene activation or inactivation by environmental impacts just before and during anthesis (Weider et al., 2009). The amount of kernels detected at each ear matched the flowering assessment data: the higher the rate of fertility, the more kernels were produced.

Generally, the CMS-effect, which is caused by a mitochondrial dysfunction, can be compensated by restorers of fertility genes (*Rf*-genes; Schnable & Wise, 1998). This compensation can result in fluctuated or fertile tassels with more or less vital pollen. This fact is well documented and explained as nucleo-mitochondrial

conflict, which leads to gynodioecy, a co-occurrence of female and hermaphroditic individuals within one population (Budar et al., 2003).

Especially for DSP2, the results of kernel production per cob underline the results found by Weider et al. (2009) for the same CMS maize hybrid, which they tested among other CMS maize hybrids of the three CMS cytoplasm. DSP2 belongs to the T-cytoplasm, which is known as providing—with exceptions—the most reliable male sterility (Weider et al., 2009). Restoration to fertility can only happen if two dominant *Rf* genes act together—*Rf1* and *Rf2*. Solely in the presence of *Rf2*, the *Rf1* allele affects the transcription of the responsible mitochondrial gene (*T-urf13*) and causes pollen fertility (Dewey, Timothy, & Levings, 1987). The influence of the *Rf1* gene can be compensated by other dominant *Rf* genes such as *Rf8* and *Rf** (Dill, Wise, & Schnable, 1997). These genes are rarely present in T-cytoplasm types. Their expression is environmentally sensitive and can result in a few fertile or partly restored plants in one population (Dill et al., 1997; Weider et al., 2009). The greenhouse results for DSP2 confirm the field-trial results of Bückmann et al. (2013) and Weider et al. (2009). Obviously, the slight dependency of DSP2 male sterility on weather conditions—which is paralleled by pollen dispersal and kernel production—is dependent on the sporophytic T-cytoplasm, as described by Dill et al. (1997). Concerning kernel production, Bückmann et al. (2013) and Weider et al. (2009) came to the conclusion that the kernel development of DSP2 increases with decreasing temperatures occurring shortly before the onset of anthesis (anther and pollen formation).

Torres and Zidane both belong to the CMS S-type. This cytoplasm is the most unstable one (Gabay-Laughnan, Chase, Ortega, & Zhao, 2004; Gabay-Laughnan, Zabala, & Laughnan, 1995) and the restoration to fertility is more complex compared to the T-cytoplasm. It is probably dependent on one dominant (not environment-sensitive) restorer gene (*Rf3*) and a large number of spontaneously occurring *Rf* genes, as well as more than 60 restoring allele mutations (Gabay-Laughnan, 1997; Gabay-Laughnan et al., 1995; Laughnan & Gabay, 1978). Gabay-Laughnan, Kuzmin, Monroe, Roark, and Newton (2009) identified the *Rf9* gene, which is expressed stronger under moderate—compared to higher—temperatures. According to the greenhouse results, an increasing level of *Rf9* gene expression with decreasing temperatures could be expected for Zidane, resulting in partly restored or fertile tassels. In the case

of Torres, the partly restored tassels produce only a very small amount of pollen.

Apart from the type of the CMS cytoplasm, the influence of the air temperature on the CMS trait was obvious and might be explained by the sensitivity of *Rf* gene activation or inactivation just before anthesis. Weider et al. (2009) tested 22 CMS maize hybrids in 17 environments. They found an influence of climate on CMS restoration, but a clear correlation of climatic influences and fertility restorations could not be found. This also matches the field-trial results of Bückmann et al. (2013), which were collected in three different German environments in 2009 and 2010. There, a higher level of sterility was measured in 2010, dependent on weather conditions: In the first half of July 2010, the average daily air temperatures increased by 3°C to 3.5°C above the longstanding average temperature at all trial locations. In general, less pollen was produced by all CMS maize hybrids tested and, consequently, mean kernel sets ([MKS] produced kernels in relation to a completely fertilized ear) values were very low as well. This indicates an influence of air temperature just before anthesis on the restoration of fertility on both T-cytoplasm and S-cytoplasm. Duvick (1965) and Tracy, Everett, and Gracen (1991) already assumed that cool and humid conditions cooperate in the restoration of fertility, whereas hot and dry conditions maintain sterility. Hot and dry weather causes the development of fewer anthers, and pollen can lose its viability or is non-competitive compared with pollen of fertile plants (Sotchenko, Gorbacheva, & Kosogorova, 2007).

The influence of high temperatures was described for other crops as well. Previously, Sarvella (1966) described in experiments with cotton that lower temperatures combined with higher humidity were associated with an increased restoration of fertility of CMS plants, but the temperature was the most important factor in this relation. Marshall et al. (1974) found that temperatures above 33°C were required for the consistent expression of the CMS trait in cotton. This not only might be linked to *Rf* expressions but might be caused by toxin production (Sakata et al., 2010), which affects the CMS trait. Apart from the influence of air temperature on the stability of the CMS trait, other environmental impacts were discussed in the literature, e.g., water-limiting conditions for sorghum (Elkonin, Kozhemyakin, & Tsvetova, 2009).

Future Prospects

CMS is a proven coexistence tool for maize. Cultivation of GM CMS maize hybrids in the frame of coexistence regimes requires improved knowledge about the influence of the environment on the CMS trait and sufficient pollination of the GM CMS plants by admixing a male-fertile and non-GM pollen donor (Feil, Weingartner, & Stamp, 2003). If the CMS maize hybrid and the pollinator plant provide a different genetic background, yield can be significantly increased (Munsch et al., 2010; Stamp, Chowchong, Menzi, Weingartner, & Kaeser, 2000; Weingartner, Kaeser, Long, & Stamp, 2002). The so-called plus-hybrid effect (Feil et al., 2003; Feil & Stamp, 2002) combines the potential benefits of CMS and a xenia effect. CMS hybrids have a “female advantage” over their male-fertile counterparts, which may be caused by increased female fertility related to the reallocation of resources unused in male function or by greater seed vitality by avoiding self-pollination (Budar et al., 2003). Previously, Kiesselbach (1960) defined xenia as the direct effect of an unrelated pollinator on the developing kernel. The xenia effect on single-kernel weight was associated with changes in the rate of kernel growth (Seka & Cross, 1995) as well as changes in the activity of specific enzymes (Bulant, Gallais, Matthys-Rochon, & Prioul, 2000).

Hence, when growing GM CMS maize hybrids, pollen containment will be combined with yield increase. Currently, it is recommended to grow 80:20 mixtures of GM CMS hybrids and male-fertile non-GM hybrids for obtaining sufficient yields (Munsch et al., 2010). There is little information about the most effective pollinator proportions. The 80:20 proportion is considered as being high enough for adequate pollination. Since the early 1990s, xenia has been applied in practice in the TopCross system (registered trademark of DuPont Specialty Grains) to influence qualitative kernel traits. This production system includes 90% of a high-yielding CMS maize hybrid mixed with 10% of a pollinator (Thomison & Geyer, 1999), which was sufficient to achieve successful oil content but grain yields were lower compared to control groups. This topic needs to be investigated with other CMS maize hybrids more related to grain yield. As well, the question of the optimal distribution of the pollinator in a crop stand remains to be answered.

Studies being performed in the frame of the EU-funded project PRICE (Practical Implementation of Coexistence in Europe)¹ will provide further data to optimize the current recommendations. One PRICE

work package is dealing with the plus-hybrid system using the CMS maize hybrid Torres in Germany, Spain, and Czech Republic. Different amounts (10%, 15%, 20%) and distributions of the pollinator (mixture, row) are tested. The studies began in 2012. The reasons for choosing Torres were its wide agricultural use, the strong reliability of the CMS trait and our own experiences with this hybrid (Bückmann et al., 2013). Due to its low FAO cycle, Torres is not the most suitable hybrid for Spain; however, since it received comparable results in different environments, it was decided to test it also in Spain in comparison with the trials in Germany and the Czech Republic.

Finally, the results will complete the recommendations for a good agricultural practice to guarantee the coexistence of cropping systems using GM plants based on CMS and non-GM plants.

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1. <http://www.price-coexistence.com>

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