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Cytoplasmic male sterility as a biological confinement tool for maize coexistence: optimization of pollinator spatial arrangement

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ABSTRACT

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Cytoplasmic male sterility (CMS) allows efficient biological confinement of transgenes if pollen-mediated gene flow has to be reduced or eliminated. For introduction of CMS maize in agricultural practice, sufficient yields comparable with conventional systems should be achieved. The plus-cultivar-system in maize offers a possibility for biological confinement together with high and stable yields whereas pollinator amount and distribution within the CMS crop is crucial. The aim of this EU-funded study was to identify the best proportion (10, 15, and 20%) and spatial arrangement (inserted rows, mixed seeds) of the pollinator within the CMS maize cultivar under field conditions in the Czech Republic, in Germany and in Spain. In Germany and in the Czech Republic, a pollinator proportion of 10% produced significantly lower yield than the treatments with a pollinator proportion of 15% and 20%. Differences in yield between row and mix arrangements were not detected. No differences between the tested arrangements occurred in Spain. With respect to practical conditions, a pollinator proportion of 15% can be recommended for achieving a satisfactory yield. CMS maize cultivar released no or merely a small amount of pollen and self-pollinated plants developed no or only a small number of kernels indicating that currently recommended isolation distances between genetically modified (GM) and non-GM fields can be substantially shortened if the CMS confinement tool is used.

Keywords: genetically modified maize; outcrossing potential; spatial distribution

For growing genetically modified (GM) maize in the European, Union the Commission of European Communities (2009) recommends national coexistence regulations to ensure the freedom of choice

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between different growing systems for farmers and consumers. This includes reliable confinement tools, especially for maize as a cross-pollinated plant, to reduce or even prevent an unintended spread of transgenes into neighbouring fields. Cytoplasmic male sterility (CMS) represents a useful and sufficient biological confinement method to achieve this goal (Bückmann et al. 2013).

CMS is a maternally inherited trait that suppresses the production of functional pollen grains (Duvick 1965, Laser and Lersten 1972, Schnable and Wise 1998). A loss-of-function mutation in the mitochondrial genome (Chase and Gabay-Laughnan 2004) entails a dysfunction of the respiratory metabolism, interfering with the male gamete production (Budar et al. 2003, Chase 2006). The female fertility of the plant is not affected. Generally, three CMS-types are known (Sofi et al. 2007), the CMS-T or Texas cytoplasm, CMS-S or USDA-cytoplasm and CMS-C or Charrua-cytoplasm. They differ in the genomic location of the trait and the presence of certain nuclear restorer genes, which can compensate the CMS effect and restore fertility (Schnable and Wise 1998).

The cultivation of GM CMS maize cultivars requires a sufficient pollination of the maternal plants by admixing a male-fertile pollen donor (Feil et al. 2003). If the CMS maize cultivar and the pollinator plant have different genetic backgrounds, the yield can significantly increase (Stamp et al. 2000, Weingartner et al. 2002, Munsch et al. 2010). CMS cultivars 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). The so-called plus-cultivar-effect (Feil and Stamp 2002, Feil et al. 2003) combines the potential benefits of CMS and a Xenia effect. Already Kiesselbach (1960) defined Xenia as the direct effect of an unrelated pollinator on the developing kernel. Different to the heterosis effect, which is mainly based on the optimization of the F1-generation in classical breeding, Xenia is related to the F2-generation (Munsch 2009).

Since the early 1990s, Xenia has been applied in practice in the TopCross system (registered trademark of DuPont Specialty Grains). This production system, developed by Thomison and Geyer (1999) to influence the qualitative kernel traits (oil content and protein quality), works with 90% of a high-yielding CMS maize cultivar mixed with 10% of a pollinator.

Hence, when growing GM CMS maize, cultivars pollen confinement can be combined with yield increase. Currently, it is recommended to grow 80:20 mixtures of GM CMS cultivars and male fertile non-GM cultivars for obtaining sufficient yields (Munsch et al. 2008). There is only little information about the most effective pollinator proportions. The 80:20 proportion is considered as being high enough for an adequate pollination.

The aim of this study is to optimize the current recommendations regarding the proportion and spatial distribution of the pollinator within a GM CMS maize cultivar under field conditions in three European environments. The results should be considered in the recommendations for 'Good Agricultural Practice' of GM crop cultivation.

MATERIAL AND METHODS

During two experimental years (2012 and 2013), field trials with CMS maize were carried out in the Czech Republic, in Spain and in Germany to identify the best proportion and distribution of the pollen donor. In 2014, additional trials were carried out for testing whether the pollinator itself has an impact on the yield. The trial locations

Table 1. Characterisation of trial localities

	Germany	Czech Republic	Spain
Location	Saxony-Anhalt, Quedlinburg, North-Eastern foothills of the Harz	Louny, Hrádek and Lenešice	Catalonia, Baix Empordà
Soil type	Loam, black soil, Lö 1a	Chernozem, Rendzina	Xerofluvent Oxiaquic
Average annual temperature (°C)	8.9	8.5	14.8
Altitude (m a.s.l.)	140	250, 200	12
Average annual precipitation (mm)	497	456	671

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varied in soil type, annual precipitation as well as total and yearly average air temperature (Table 1).

Different arrangements (mixed seeds and inserted rows) and different pollinator proportions (10, 15, and 20%) within the CMS cultivar were tested. The plots had a size of 15 × 15 m which resulted in 20 rows per plot with a row distance of 0.75 m grouped in three randomized blocks. The Spanish trial was conducted slightly differently than in to the Czech and German ones (no groups). In 2014, only row arrangement trials for testing the influence of the pollinator on yield were performed.

The CMS-type of the commercial cv. Torres (KWS, Germany) was chosen because of its low cross-pollination potential (Bückmann et al. 2013). A white maize (WM) cultivar, DSP 17007, from the Delley Seeds and Plants Company, Switzerland, was grown as pollen donor in all plots in 2012 and 2013 and in one variant in 2014. This cultivar was chosen due to similar growing dynamics as cv. Torres. In 2014, DSP 17007 was replaced by cv. Grosso (KWS, Germany). For checking purposes, the former pollinator DSP 17007 was tested in parallel with 15% pollinator proportion.

To avoid pollen flow from one plot to another, hemp (Germany) and sorghum (the Czech Republic and Spain) were grown as buffer crops between the plots. Soil preparation, crop protection and fertilization were performed according to local recommendations to achieve a high quality grain yield.

When the CMS maize cv. Torres developed anthers and pollen in 2012 and 2013, self-pollinations were carried out to test the fertility of the CMS pollen. This procedure was executed by hand on 10 plants per plot in Germany and in the Czech Republic. In Spain, a separate plot with cv. Torres was grown so that the plants pollinated themselves. The developed kernels were counted. The number of kernels of each cob and the total number of kernels of a completely fertilized cob were used to calculate the mean kernel set (MKS).

In Germany and in the Czech Republic, the plots were harvested row by row. All row yields were summarized afterwards. In Spain, two rows were harvested together. The kernels were weighed, dried and the yields were calculated to 14% grain moisture.

The results were statistically evaluated by the analysis of variance with post-hoc Scheffe's test ($P = 0.05$) using the Origin 8.1G software (OriginLAB, Northampton, USA).

RESULTS AND DISCUSSION

Cv. Torres belongs to the CMS-S type which can restore fertility (Gabay-Laughnan et al. 1995). The sterility of CMS-S type cultivars such as cv. Torres is known as being unstable (Gabay-Laughnan et al. 1995, Gabay-Laughnan 1997). This fact was observed at all three trial locations. Cv. Torres developed partly restored tassels and produced a small amount of pollen. As it was intended, the flowering times of cv. Torres and the pollen donors DSP 17007 and cv. Grosso overlapped and were coincident at all locations and experimental years.

Self-pollination by hand was carried out in the Czech Republic and in Germany in 2012 and resulted in both unfertilized ears with no developed kernel and some ears with a reduced number of kernels. Hence, the mean kernel set values equalled 0.93% (± 1.29%) in Germany and 4.59% (± 7.77%) in the Czech Republic in relation to a fully fertilized cob. In Spain, a self-pollination plot was conducted in 2012 showing similar results (MKS: 13 ± 7.4%). Due to a longer pollination period in the plot (no isolation of tassels and ears), the number of developed kernels was clearly higher than in manually pollinated plants.

The fact that CMS-S maize cultivars can develop fertile pollen needs to be taken into account when a GM CMS maize cultivar is cultivated and cross-pollination needs to be controlled. Nevertheless, the cross-pollination rate of cv. Torres was determined to be very low (< 0.2% at 3.50 m distance from the Torres field) in large-scale field trials at different locations in Germany. Compared to fully fertile maize cultivars, CMS maize cultivars can provide a reduction of cross-pollination up to 100% (Bückmann et al. 2013). Thus, efficient coexistence strategies can be developed by exploring the CMS trait. Furthermore and in line with current European coexistence recommendations based on a GM admixture threshold of 0.9%, the data of Bückmann et al. (2013) argue for a drastic reduction of the isolation distance between neighbouring GM and non-GM maize fields when CMS maize is used. Thus, the potential costs of coexistence would also be reduced because isolation distances are one of the most cost intensive coexistence measures and the isolation distance correlates with the correspondent costs (Venus et al. 2016). A comprehensive review about coexistence policies in the EU member states is given in Schenkelaars and Wesseler (2016).

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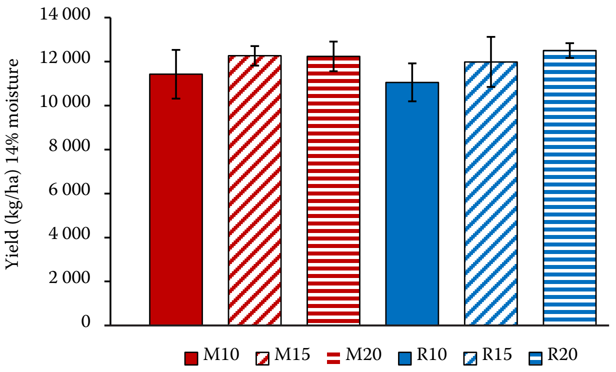

Figure 1. Grain yield with different pollinator proportions and spatial arrangements – the 2012 field trial in Germany as a representative example. Arrangements: M – mix; R – row; proportions: 10 – 10% pollinator; 15 – 15% pollinator; 20 – 20% pollinator). Vertical bars express standard deviation

All trials could be harvested successfully in Germany. In the Czech Republic, the cultivar 15% row of the 2012 trial and the entire 2013 trial had to be taken off the calculation due to an impaired plant development in two replications caused by flooding and due to wild boar damage shortly before the harvest respectively. An unusual hailstorm during the flowering period in 2013 in Spain might have negatively affected grain yield.

In Germany, grain yields were significantly lower when the 10% pollinator proportion (cv .WM) was used compared to 15% and 20% (Figure 1) in 2012, but not in 2013 (Table 2), when the yield showed similar level without significant differences for all tested proportions. Row and mix arrangements of the pollinator did not differ in yield. In 2014, a different pollinator (cv .Grosso) was used in row arrangement plots, confirming the results from 2012 and 2013.

The significantly lower yields in the 10% pollinator proportion were confirmed by the results from the Czech Republic in 2012 and 2014 whereas no significant differences could be observed between the 15% and 20% pollinator scenario (Table 2). Thus, the proportion of the pollinator should be higher than 10% but does not need to exceed 15%. The trials in Spain showed no significant differences between tested variants. This and the unusual low average yield in Spain might be explained by the fact that cv. Torres, as well as cvs. WM and Grosso, have a FAO cycle as low as 280, so they are not adequate for the Spanish climate conditions.

The harvest technique 'row by row' allowed a comparison of the yield of the pollinated CMS maize cv. Torres and the cv. WM pollinator. At all three locations, WM had a lower yield than Torres (Figure 2), reducing the yield from the plot. For this reason, field trials with the conventional pollen fertile and widely commercialized maize cv. cv. Grosso (Table 3) in row arrangements were car-

Table 2. Grain yields (kg/ha) for different proportions and spatial arrangements of the cvs. WM (2012 and 2013) and Grosso (2014) pollinators

Year	Variables		Germany	Czech Republic	Spain
2012*	arrangement	mix	11 963^{a}	7986^{a}	9358^{a}
		row	11 847^{a}	7641^{a}	9615^{a}
	percentage	10	11 234^{a}	7179^{a}	9269^{a}
		15	12 109^{b}	8118^{b}	9777^{a}
		20	12 361^{b}	8794^{b}	9414^{a}
2013**	arrangement	mix	11 239^{a}	–	8992^{a}
		row	11 350^{a}	–	8657^{a}
	percentage	10	11 095^{a}	–	9330^{a}
		15	11 350^{a}	–	8835^{a}
		20	11 438^{a}	–	8445^{a}
2014***	percentage	10	12 586^{a}	6575^{a}	7047^{a}
		15	12 666^{b}	7763^{b}	7278^{a}
		20	12 997^{b}	8593^{b}	7443^{a}

Multiple ANOVA test was calculated for each locality separately. The same letters for homogenous groups indicate that the mean values do not differ significantly ($P = 0.05$). *variant row 15% excluded (flood); **trial in the Czech Republic excluded (wild boar damage); ***pollinator changed in 2014: cv. Grosso instead of DSP17007; only row arrangements were tested

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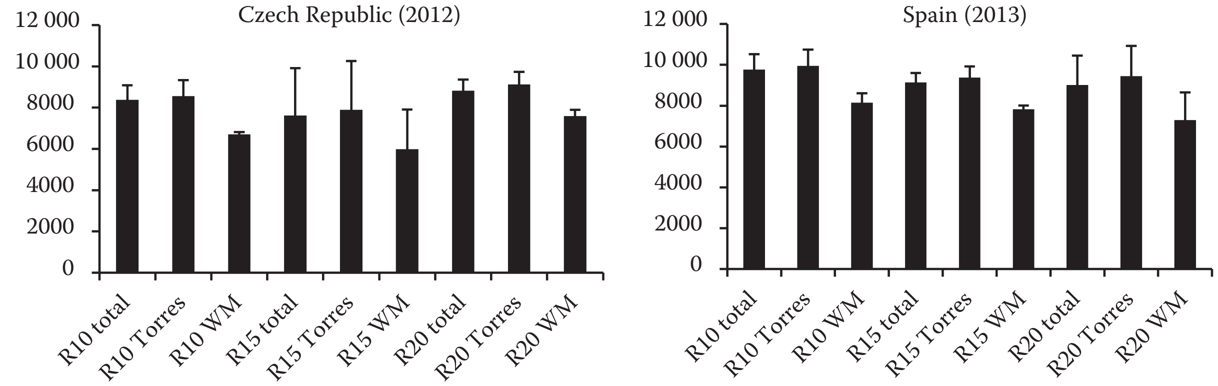

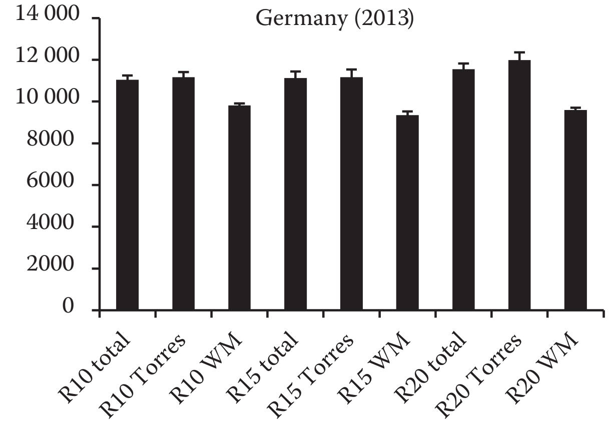

Figure 2. Average grain yields calculated with and without pollinator rows. Arrangements: M – mix; R – row; proportions: 10 – 10% pollinator; 15 – 15% pollinator; 20 – 20% pollinator; cultivar yield; total – cv. Torres and cv. WM (white maize) together). Vertical bars express standard deviation

ried out in Germany, in the Czech Republic and in Spain in 2014. The 15% pollinator proportion was used for comparison with the trials performed in 2012, 2013 and 2014.

In the 2014 trials, the total yield was positively influenced by the use of cv. Grosso instead of cv. WM pollinator (Table 4). This is also representatively shown for the Czech Republic in Figure 3.

Table 3. Yield comparison of cvs. WM (white maize), Grosso (pollinators) and Torres (cytoplasmic male sterility pollen acceptor)

Location	Cultivar	Yield (kg/ha)
Germany	Grosso	13 823^{a}
	WM	9630^{b}
	Torres	12 570^{c}
Czech Republic	Grosso	9447^{a}
	WM	5994^{b}
	Torres	7057^{c}
Spain	Grosso	8993^{a}
	WM	2571^{b}
	Torres	7272^{c}

The same letters for homogenous groups indicate that the mean values do not differ significantly at $P = 0.05$

A 10% pollinator proportion was significantly lower in yield than 15% and 20% in Germany and in the Czech Republic in all trial years (Table 2). Admixing of 15% cv. WM resulted in significantly lower yields than admixing of 15% cv. Grosso in Germany. This was also observable in Spain and in the Czech Republic by trend (Table 4). As expected, the average yield of the single maize

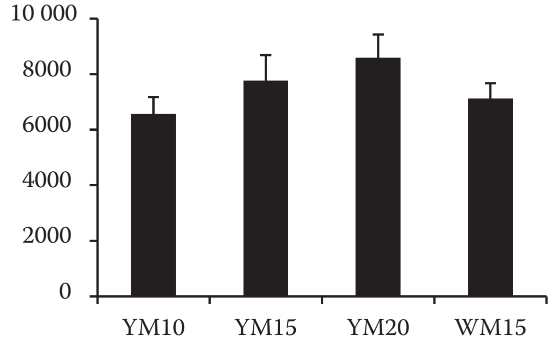

Figure 3. Yield of row arrangement with the yellow maize cv. Grosso pollinator (YM, 10, 15 and 20%) in comparison to the white maize DSP17007 pollinator (WM, 15%), calculated for 14% grain moisture (Czech Republic 2014). Vertical bars express standard deviation

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Table 4. Average grain yields calculated with and without pollinator rows

Locality	With Pollinator	Yield (kg/ha)	Without pollinator (cv. Torres only)	Yield (kg/ha)
Germany	Grosso	12 666^{a}	ex Grosso	12 443^{a}
	WM	12 173^{b}	ex WM	12 672^{a}
Czech Republic	Grosso	7762^{a}	ex Grosso	7354^{a}
	WM	7123^{a}	ex WM	7321^{a}
Spain	Grosso	7278^{a}	ex Grosso	7631^{a}
	WM	6606^{a}	ex WM	7645^{a}

The same letters for homogenous groups indicate that the mean values do not differ significantly at $P = 0.05$

cultivars showed a significant advantage of cv. Grosso compared to cv. WM (Table 3). When the pollinator rows were excluded from the calculation, both 15% variants resulted in similar yields at all locations (Table 4).

The field trials in Germany, in the Czech Republic and in Spain aimed at clarifying the question regarding the pollination requirements of GM CMS maize cultivars when cultivated for coexistence purposes.

In the presented study, 10% of a pollinator was too low for a sufficient kernel yield compared to 15% and 20% in Germany and in the Czech Republic. Quality parameters, as focused on in the TopCross systems, were not tested. The trials clearly demonstrated that there is no need to exceed a 15% pollinator proportion since a further increase did not cause an increased grain yield. In this case a potential cross-pollination risk would be drastically reduced to 15% or to zero compared to conventional maize, dependent from the choice of GM or non-GM maize as pollinator. Herbicide tolerant (HT) crops require a HT pollinator otherwise the herbicide would destroy the latter. If the CMS system is used for e.g. insect resistance (*Bt*) GM maize, a non-GM pollinator can be implemented. Following this scenario, the non-GM pollinator can create an insect refugium of 15%, which may help prevent the selection of *Bt* toxin-resistant insect populations and may render an additional sowing unnecessary (Feil et al. 2003). To avoid cross-pollination, Plus-Hybrid fields can be combined with other coexistence tools like border rows and/or isolation distances.

The trial results showed no difference between row and mix arrangements of the pollinator plants. This is an advantage for maize growers using common sowing machines.

No differences between the treatments were measured in Spain. It is likely that the FAO number of 280 was too low for Spain where maize cultivars of FAO 500 to 700 are mostly cultivated. Unfortunately, it was not possible to obtain CMS maize cultivars with these FAO cycles for the Spanish conditions. The total yield of Plus-Hybrid cultivations can be influenced positively or negatively by the specific yield of the pollinator as seen in all field trials with cv. WM. There is only little information about the total yield of CMS/pollinator-maize cultivation available in the scientific literature. The TopCross system with pollinator proportions of 8% to 10% used for high-oil corn production in the USA resulted in lower kernel yields than those with comparable cultivars (Thomison et al. 2002). European results of Plus-Hybrid studies were based on trial designs that excluded the pollinator from the harvest plot (Weingartner et al. 2004, Munsch et al. 2008, 2010).

In the presented study, the specific yield of the pollinator DSP 17007 was lower than the one of cv. Torres, whereas the yield of cv. Grosso was much higher. For instance, in 2014, the specific yields of cv. WM, cvs. Torres and Grosso amounted to 5994 (± 241) kg/ha, 7057 (± 1004) kg/ha and 9447 (± 1814) kg/ha, respectively.

When the pollinators were excluded from the yield assessment, which was possible in row arrangement variants, the effect of the pollinators DSP 17007 and cv. Grosso on yield was similar. Weingartner et al. (2002) stated that the pollination with DSP 17007 causes decreasing yields compared to pollination with the isogenic line of the CMS maize cultivar but these results were achieved with a different methodology.

Field trials with non-pollinating CMS maize in Germany, in the Czech Republic and in Spain demonstrate the performance of this biological confinement tool for coexistence of GM and non-GM production systems. The use of non-

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GM pollinator can ensure sufficient yields while strongly reducing GM pollen spread. Based on the presented results, there is no need to exceed the recommended pollinator proportion of 15%.

The results also indicate that the current isolation distances set up by most member states in their co-existence rules can be shortened if the CMS confinement tool is used and unnecessary costs and burden to farmers can be reduced.

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