

POLLEN GERMINATION AND POLLEN TUBE GROWTH IN ZP MAIZE LINES

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The study was conducted on the *in vitro* pollen germination at 26°, 28°, 32°
and 35°C for 24h of male parental lines, pollen tube growth *in vivo* in cross pollination
of female and male parental lines that make couples in four hybrids: ZP 504 su (♀
ZPPL 51 × ♂ ZPPL 67); ZP 677 (♀ ZPPL 17 × ♂ ZPPL 201); ZP 704 (♀ ZPPL 109 ×
♂ ZPPL 79), ZP 611 k (♀ ZPPL 126 × ♂ ZPPL 105), and the open pollination of
female parental lines of the above mentioned hybrids. Pollen germination *in vitro* and
pollen tube growth dynamics *in vivo* showed different genotypic specificities with the
tests applied. The obtained results were discussed in the context of reproductive biology
of ZP maize lines and aimed to create the preconditions for successful management and
direction of the process in practice - seed production in certain environmental
conditions.

Key words: parental lines; pollen; pollination; pollen tube growth, *Zea mays* L.

INTRODUCTION

Maize (*Zea mays* L.) is monoecious and alogamous species whose pistils of one plant
are fertilized with the pollen of another, within the same crop. Dusting of pollen starts in the
middle of the main branch of the tassel, extending to the lateral branches. An individual tassel
may shed pollen for 2 to 10 days, depending on the genotype and environmental conditions

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(FONSECA *et al.*, 2003). After anthers dehiscence pollen grains contain about 80% water, quickly losing its viability, especially when water content falls below 40%; it means that pollen of maize is highly sensitive to drought conditions (STRACHAN, 2004). Air humidity has different, but equally important consequences. Under conditions of relatively high air humidity pollen viability decreases to 58% within a period of one hour (LUNA *et al.*, 2001). In these conditions, anthers do not open and pollen remains closed in them, which may later have an effect on a premature loss of its vitality. Also, in conditions when temperatures are above 35°C over a period of several hours, the possibilities for successful fertilization have drastically reduced (FARRELL and O'KEEFFE, 2007). High temperature stress at the time of anthesis could have consequences on the tassel and pollen, in terms of a large reduction of their viability.

Usually two to three days after beginning of the pollen dusting, silks emerge from the whorl of husk leaves at the end of the ear. Silk elongation was most rapid during the first day of exposure, declined progressively with time, and ceased completely within 9 to 11 days (BASSETTI and WESTGATE, 1993). The elevated peroxidase activity in pollinated silk tissue indicates a specific function in pollination events (HADŽI-TAŠKOVIĆ ŠUKALOVIĆ *et al.*, 2009). Hand pollination of exposed silks with excess pollen indicated that 4 to 8 days of silk emergence were required for maximum kernel set, depending on genotype (BASSETTI and WESTGATE, 1994). Pollen grains germinate immediately after settling on the silk. The pollen tubes need between 12 and 24 hours to reach and fertilize the ovule (SLEPER and POEHLMAN, 2006). However, if the silk does not appear at the right time, it can have an adverse effect on the success of the fertilization process. In addition, under conditions of water stress pollen tubes growth is slower, and possibility for the successful fertilization decreases. The number of ovules that will develop into kernels ranges from 300-1,000, and is dependent on the genotype and factors occurring later in the development. The amount of water that is available affects the silk growth, the length of its receptivity, as well as the smooth growth of pollen tubes and subsequent fusion of the gametes. If drought is severe during the pollination period, fertilization in a number of ovules does not occur.

This study involves the examination of pollen germination *in vitro* and pollen tubes growth *in vivo* – the progamic phase of fertilization, in crosses of female and male lines - parents of four maize hybrids developed in Maize Research Institute - Zemun Polje, i.e. ZP 504 su, ZP 677, ZP 704 and ZP 611k. Pollen tubes growth *in vivo* in open pollination of these female parental lines has also been included. Investigation of maize reproductive biology aims to create the preconditions for successful management and guidance of the process in specific agroecological conditions. In practice so far, the seed production of some of these hybrids had the problems related to the regularity of process of pollination and fertilization.

MATERIALS AND METHODS

Plant materials

The research included female and male parental lines of the following hybrids: ZP 504 su (♀ZPPL 51 × ♂ZPPL 67); ZP 677 (♀ZPPL 17 × ♂ZPPL 201); ZP 704 (♀ZPPL 109 × ♂ZPPL 79), and ZP 611 k (♀ZPPL 126 × ♂ZPPL 105). The trial was established at the experimental field of the Maize Research Institute - Zemun Polje, in 2013. Female and male parental lines of all four hybrids were seeded on 5 individual plots. Each plot area was of 4 square meters (one row), and represented one replication. Each row was with 20 plants, i.e. 100

plants of each genotype of the female parental line, and 50 plants of each genotype of male parental line.

Pollen tube growth in vitro

At the time of the anther dehiscence in the central branch of the tassel, pollen was taken for the testing of pollen germination *in vitro*. Pollen of each male parental line was plated in Petri dishes on nutrition medium with 15% sucrose and 0.6% bacto-agar supplemented with 0.03% $\text{Ca}(\text{NO}_3)_2$ and 0.01% H_3BO_4 . The Petri dishes were incubated in controlled temperature chambers at 26°, 28°, 32° and 35°C for 24h. The number of germinated pollen grains and the length of their pollen tubes was determined under a Leica DM LS microscope, using software Leica IM 1000 (Image Manager). Pollen grains were counted in two Petri dishes, with three fields of views per dish. Pollen grains that germinated exceeding their radius were considered as germinating grains.

Pollen tube growth in vivo

To investigate the dynamics of the pollen tubes growth in the proximal part of the silk and ovary, ears of which have not yet occurred silk were isolated, in the following female parental lines: ZPPL 51, ZPPL 17, ZPPL 109 and ZPPL 126. At the same time, in order to collect pollen, in four male lines: ZPPL 67, ZPPL 201, ZPPL 79 and ZPPL 105, the tassels without pollen dusting were isolated. In the period of young silk, 1-2 days after its occurrence, and medium old silk ($5 \pm 1-2$ days after appearance), isolated ears of female parental lines were pollinated with the pollen of male lines, in accordance with appropriate parentage that make hybrids ZP 504 su, ZP 677, ZP 704 and ZP 611 k. Subsequently, pollinated ears were isolated again with paper bags, to prevent uncontrolled pollination. 15-30 ovaries with silks of each treatment were fixed on the first and third days after pollination in FPA (70% ethanol, propionic acid and formaldehyde, 90:5:5 percentage by volume), using random sampling from the basal, medial and upper part of the ear. In the same way and in the same terms, samples were taken from the open pollination, in all four female parental lines.

To investigate pollen tubes growth in silk and ovary, a method of aniline blue staining was used (PREIL, 1970). The separation of the proximal part of silk from the ovary, and the opening of the ovary under stereo microscope have been done. Proximal parts of the silks have squashed, in order to make histological preparations. Pollen tubes in silk and ovary were monitored under UV light on the LEICA DM LS microscope, filter A (340-380 nm). The number of pistils with pollen tubes in their certain parts was also determined, in both pollination variants.

Examination of air temperature and humidity

During the field experiment, daily air temperatures and humidity were monitored during June and July 2013, using PCE-FWS 20 Easy-Weather Station, Version 6.2.

Statistical analysis

The data were statistically analyzed using one factor analysis of variance (ANOVA). In the results expressed in percentages, the arcsin square-root data transformation was performed. The significance of differences among mean values was determined by Duncan's multiple range test at $P \leq 0.05$. Statistical analyses were performed using SPSS statistical software package, Version 8.0 for Windows (SPSS. Inc., Chicago, IL).

RESULTS

Pollen germination in vitro

Test pollen germination *in vitro* is one of the main indicators of pollen functional viability. In the average for all temperatures, and individually at each constant temperature, the highest percentage of pollen germination was recorded in ZPPL 201 (50.2%) and the lowest in ZPPL 105 (12.2%) (Fig. 1 and Tab. 1). It has been found that there are significant differences between the male lines in terms of pollen germination and pollen tube length at different temperatures. Lines ZPPL 79 and ZPPL 105 had the highest pollen germination at lower temperature of 26°C i 28°C. In contrast, pollen of ZPPL 67 and ZPPL 201 lines had the highest germination percentage at 32° and 35°C.

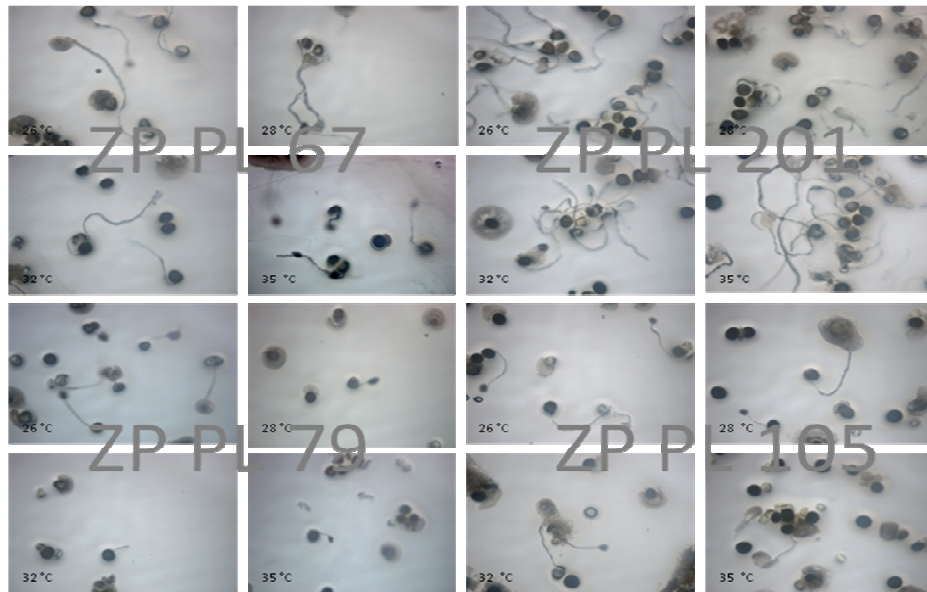


Figure 1. Pollen germination *in vitro* in four male parental lines of maize: ZPPL 67, ZPPL 201, ZPPL 17 and ZPPL 105 at 26°, 28°, 32° and 35°C

In terms of the length of pollen tubes, average for all temperatures, ZPPL 201 had the highest length of pollen tubes (673.3 μm), while the shortest was in ZPPL 79 (267.3 μm). In ZPPL 67, ZPPL 79 and ZPPL 105 lines, the longest pollen tubes were measured at 26° and 28°C. In these lines the percentage of pollen germination was accompanied by corresponding length of pollen tubes at the same or adjacent temperatures. Contrary to this, the percentage of pollen germination and pollen tube length in ZPPL 105 line showed the largest differences - at the highest temperature, the percentage of pollen germination was the lowest (8.5%), while at the lowest temperature pollen tubes had the shortest length (383.2 μm).

Table 1. Pollen germination and pollen tube length *in vitro* at different temperatures

Temperature	ZPPL 67 (male line for hybrid ZP 504 su)	
	Pollen germination (%)	Length of pollen tube (μm)
26° C	48.1 \pm 0.7 ab	476.0 \pm 11.2 b
28 °C	46.6 \pm 0.8 b	668.1 \pm 25.6 a
32 °C	52.0 \pm 0.7 a	486.5 \pm 10.8 b
35 °C	47.6 \pm 0.6 b	473.1 \pm 5.6 b
Average	48.6 \pm 0.7	525.9 \pm 13.3

Temperature	ZPPL 201 (male line fo□ hybrid ZP 677)	
	Pollen germination (%)	Length of pollen tube
26° C	49.4 \pm 0.4 b	906.4 \pm 1.5 a
28° C	41.0 \pm 0.4 c	684.2 \pm 5.1 b
32° C	42.9 \pm 0.2 c	586.1 \pm 6.1 c
35 °C	67.6 \pm 0.9 a	517.3 \pm 5.3 d
Average	50.2 \pm 0.5	673.3 \pm 4.5

Temperature	ZPPL 79 (male line for hybrid Z□ 704)	
	Pollen germination (%)	Length of pollen tube (μm)
26°C	20.7 \pm 0.6 a	334.9 \pm 22.8 a
28 °C	16.1 \pm 1.2 bc	222.2 \pm 4.9 c
32 °C	12.6 \pm 1.0 c	216.8 \pm 15.9 c
35 °C	19.2 \pm 0.8 ab	295.3 \pm 14.1ab
Average	17.2 \pm 0.9	267.3 \pm □4.4

Temperature	ZPPL 105 (male line for hybrid ZP 611k)	
	Pollen germination (%)	Length of pollen tube (μm)
26°C	18.5 \pm 0.2 a	383.2 \pm 5.8 d
28 °C	11.6 \pm 0.7 b	593.1 \pm 12.9 a
32 °C	10.1 \pm 0.1 c	410.6 \pm 3.3 b
35 °C	8.5 \pm 0.2 d	408.9 \pm 4.2 b
Average	12.2 \pm 0.3	449.0 \pm 6.6

*Within each column, the same letters indicate no significant difference at the $P \leq 0.05$ according to Duncan's multiple range

Pollen tube growth in the silk

Monitoring of the dynamics of pollen tube growth in the silk provided an opportunity to make a better assessment of the effectiveness of pollinators in the certain environmental conditions. Within a few minutes after adhesion on the trichomes, the pollen grains start to initiate pollen tubes (Fig. 2a). Inside the tissue of silk pollen tubes show strong color reaction with aniline blue fluorochrome, so that they can be clearly distinguished from the vascular tissue in which fluorochrome also accumulated. One or more of the pollen tubes was observed in the proximal end of the silk and ovular cavity in both cross- and open pollination variants (Fig. 2b, c, d, e). Although the applied histological technique does not allow visualization of the embryo sac, it was seen in several cases (Fig. 2f).

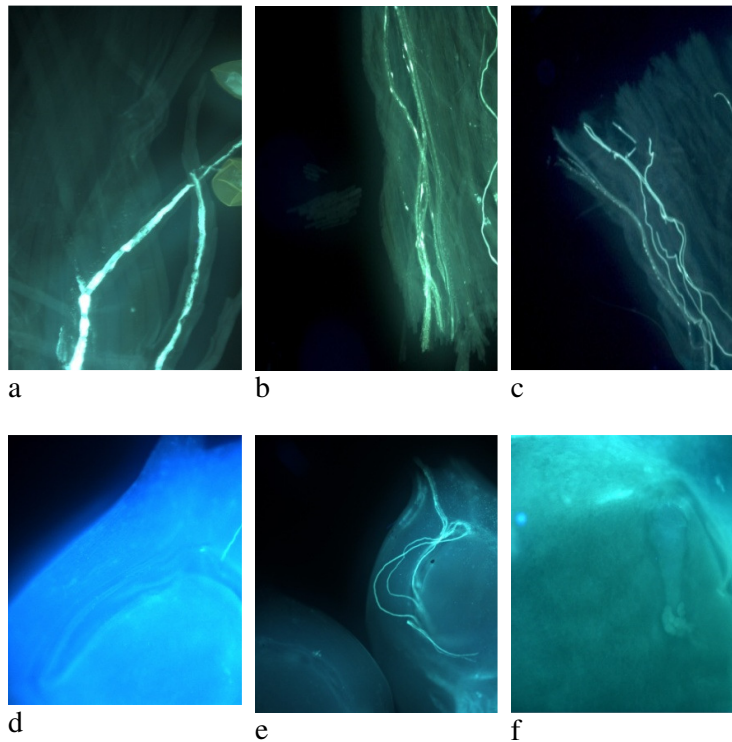
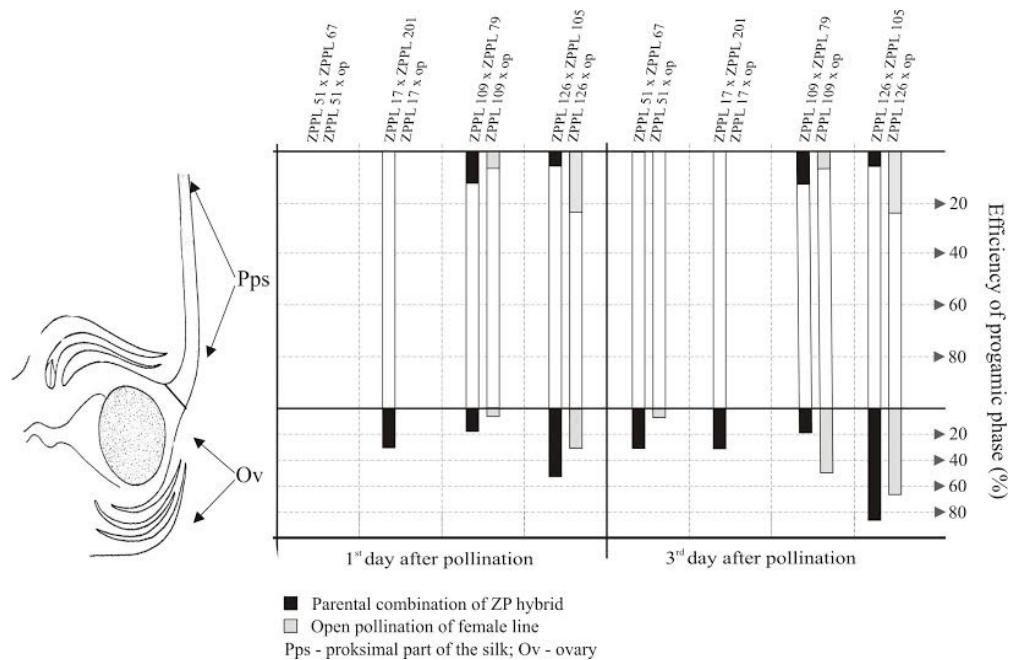


Figure 2. Pollen germination into silk trichoma (a). Pollen tube growth in proximal part of silk (b). Several pollen tubes in proximal part of silk (c). One (d) or several pollen tubes (e) penetrating ovary. Embryo sac structure in the nucellus of ovule (f)

The largest number of pistils with pollen tubes penetrating the ovary was recorded in the ZPPL 126 × ZPPL 105 cross, in the first and third day after pollination, in young silk (52.6%;

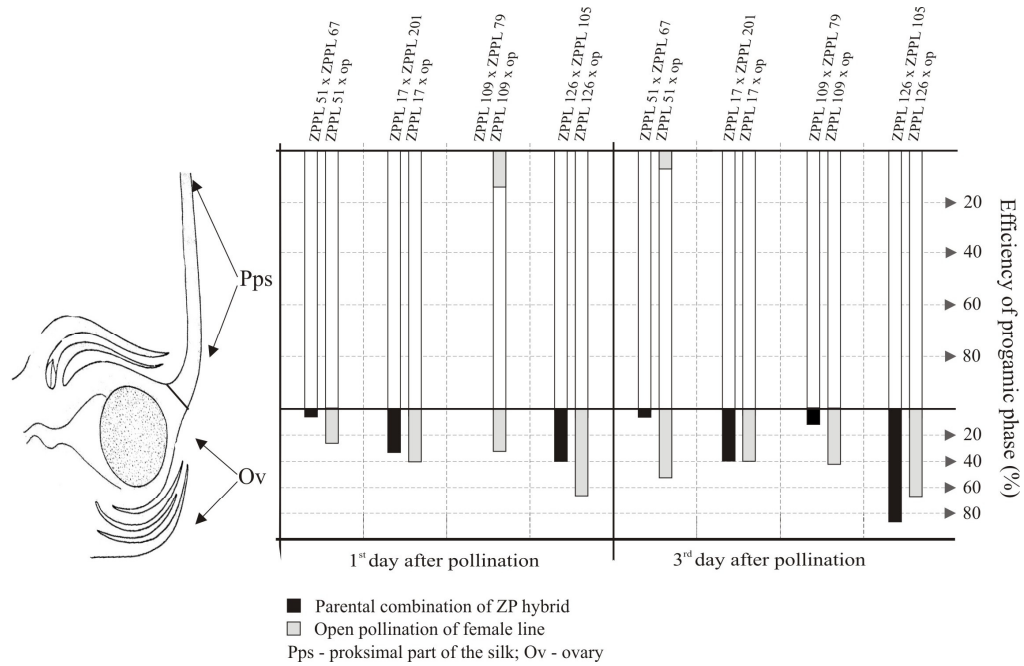
87.5%, respectively) (Graph 1). Otherwise, in the young silk male pollen was more effective in cross pollination compared to the open pollination. The only exception was the ZPPL 109 × ZPPL 79 cross, where in the third day after pollination, penetration was observed in 50% of the pistil pollen tubes.



Graph 1. Dynamics of pollen tube growth through certain pistil parts – young silk in female parent lines of four maize hybrids in cross- and open pollination

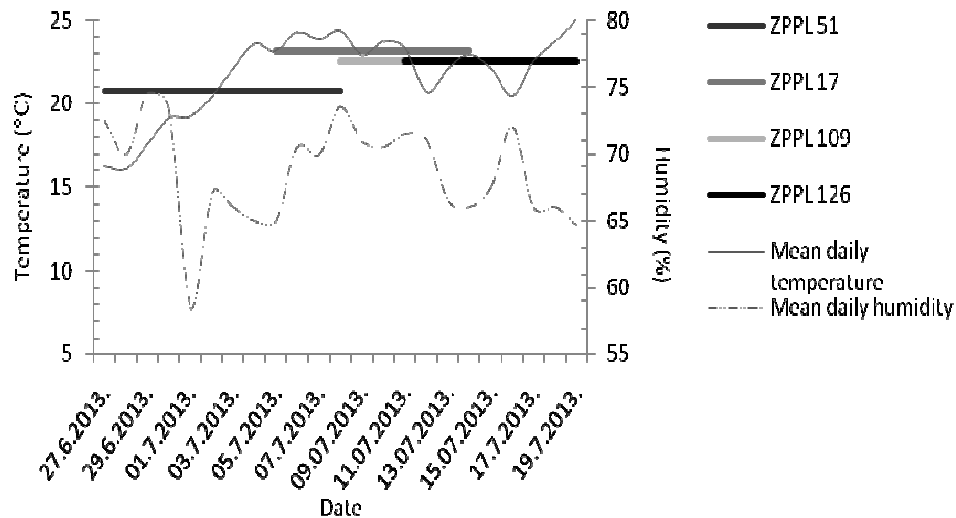
Situation is somewhat different in the middle aged silk (Graph 2). In the first day after pollination, open pollination variant showed greater efficiency of pollen tube growth. In the third day after pollination, open pollination was more efficient in only two female lines (ZPPL 51 and ZPPL 109). Cross ZPPL 126 × ZPPL 105 was particularly interesting, showing a faster growth of pollen tubes in the first day after pollination. In the third day after pollination, this cross reached the penetration value as in young silk.

The results showed that the growth of the pollen tubes was more efficient in the middle aged than in young silk. The penetration of pollen tubes in the ovary was observed in all tested combinations.



Graph 2. Dynamics of pollen tube growth through certain pistil parts – middle aged silk in female parental lines of four maize hybrids in cross- and open pollination

Mean daily temperature, average mean daily temperatures and relative humidity during pollination of young and middle old silk in all four female lines are given in Graph 3. The mean daily temperature during the indicated period was 20.8°C for the cross ZPPL 51 × ZPPL 67 (ZP 504 su). This combination starts early with pollination. In the crosses ZPPL 17 × ZPPL 201 (ZP 677) and ZPPL 126 × ZPPL 105 (ZP 611 k), average mean daily temperatures were 23.1°C and 22.5°C, respectively. The cross ZPPL 109 × ZPPL 79 (ZP 704) was the latest among the all tested crosses, whereas the average daily temperature was 22.5°C. The average mean daily temperature for the period of pollination and fertilization of the above-mentioned parental lines of ZP hybrids ranged from 16.2° to 25.1°C, while the average relative humidity ranged from 58.5 to 74.5%.



Graph 3. Average daily temperature and humidity as well as the average mean daily temperature in the field during the period of pollination of four female parental lines of hybrids

DISCUSSION

Several factors influence on kernal set in maize, among which are the emergence of silk, pollen shedding, pollen viability, female receptivity, fertilization success and environmental conditions. In all four male parental lines, a large variability in terms of pollen germination *in vitro* as noticed with other maize genotypes in approximately the same experimental conditions have been found (SCHOPER *et al.*, 1987). Based on the results of pollen germination *in vitro*, the best pollen germination was recorded in the male parental lines: ZPPL 67 and ZPPL 201. These lines showed good germination at higher temperatures of 32° and 35°C. Previous studies pointed out that maize pollen rapidly loses its vitality at the higher temperatures of 38° and 40°C (WESTGATE, 2004). Otherwise, the structure and physiology of pollen is under genetic control which together with the composition of the medium directly affects the germination and pollen tubes growth *in vitro* (GEETHA *et al.*, 2004). The results of this study indicate that different genetic backgrounds of male paternal lines showed different *in vitro* germination characteristics. In this experiment, a constant temperature for growth *in vitro* were higher compared with the average daily temperatures measured in the open field and can serve as an indicator of possible pollen germination of these genotypes, if such temperature conditions predominate.

Pollen tubes growth *in vitro* usually do not reach the length that would be required to affect fertilization in nature. In maize, pollen grain germination and growth of pollen tubes *in vivo*, i.e. the progamic male gametophyte development, can be divided into five separate stages (SWANSON *et al.*, 2004). Similarly to other plants, the first three phases - adhesion of pollen grain

on the stigma surface (trichome cells), pollen tube growth initiation, and its conducting within the transmitting tract of the style, are guided by anatomical aspects of the trichome cells and style, and also the pollen-stigma interaction and signaling (LAUSSER *et al.*, 2010). Pollen tubes attraction and growth supported by the transmitting tract seem to play key roles in progamic pollen tube growth. In this study, pollen tube growth *in vivo* was always observed near the vascular bundle, which is the source of water and nutrients essential for growth (STRACHAN, 2004). In the fourth stage of the progamic phase, pollen tubes from transmitting tract entering in the ovarial cavity and grow at the surface of inner integument towards the micropylar region (CEROVIĆ *et al.*, 1999). In our research, we determined the penetration of more than one pollen tubes in the ovary. Otherwise, in these regions, control of pollen tubes growth by the female gametophyte was revealed (MARTON *et al.*, 2005; DRESSELHAUSE and MARTON, 2009). Penetration of pollen tubes in the proximal part of the silk and ovary was observed in almost all combinations already after 24 hours of pollination. Different efficiency of pollen tube growth in different pollination variants is closely related to genotype specificities included appearance of incompatibility (BOŠKOVIĆ *et al.*, 2006; SUTHERLAND *et al.*, 2009). The results are a direct demonstration that pollen competitive ability, measured by pollen tubes growth rate, depends to a considerable extent on genes expressed in both tissues.

Although the *in vivo* results generally agreed with *in vitro* results, they indicated that *in vitro* germination capacity and fertilization ability were not completely related. The gene expression pattern of pollen tubes grown *in vitro* differ from that of pollen tubes grown through the stigma and style (QIN *et al.*, 2009). Thus, in our research, male paternal lines with the highest average germination and pollen tube length *in vitro* (ZPPL 67 and ZPPL 201) had a weak dynamics of pollen tube growth *in vivo*, in relation to the ZPPL 105 line. The degree of the difference between the lines was however changed, presumably because pollen-style or pollen-pollen interactions are absent *in vitro*.

That is why pollen with low germination rate *in vitro*, can produce satisfactory seed set *in vivo*, and vice versa, so the most accurate test of pollen viability is the ability of pollen grain to effect fertilization and seed set (SHIVANA and JOHRI, 1985).

The maximum number of grain sets at ample pollen supply and one pollination date was usually attained 3 to 5 days after the emergence of silk and started to decrease at 7 days silk (KAESER *et al.*, 2003). Our results indicate that the status of silk (young or middle aged) was not a limiting factor for pollen tube growth dynamics in all examined combinations. Although our work is not accompanied by kernel set investigation, in some papers it was stated that for the kernel set in an open-pollinated field, up to 7 days of pollination would be required (STRUİK *et al.*, 1986).

In current seed production, impact of agro-ecological factors was observed in some years and locations (PAVLOV and CREVAR, 2014). The results obtained in this paper, related to the examination of pollen germination *in vitro* and *in vivo* in parental lines of hybrids ZP 504 su, ZP 677, ZP 704 and ZP 611 k are the first in the field of pollination and fertilization of the ZP hybrids lines in certain agroecological conditions in our country. In practical terms, studies on reproductive biology could serve to develop and test prediction models concerning the impact of genotype and environmental factors such as temperature and humidity on the effectiveness in maize seed production.

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KLIJAVOST POLENA I RAST POLENOVIH CEVČICA KOD ZP LINIJA KUKURUZA

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Izvod

U ovom istraživanjima ispitivani su klijavost polena *in vitro* i rast polenovih cevčica *in vivo* muških roditeljskih linija u kombinacijama oprašivanja roditeljskih parova kod četiri hibrida kukuruza: ZP 504 su (♀ZPPL 51 × ♂ZPPL 67); ZP 677 (♀ZPPL 17 × ♂ZPPL 201); ZP 704 (♀ZPPL 109 × ♂ZPPL 79), i ZP 611 k (♀ZPPL 126 × ♂ZPPL 105), i slobodnog oprašivanja majčinskih linija navedenih hibrida. Na osnovu ispitivanja klijavosti polena *in vitro* i dinamike rasta polenovih cevčica *in vivo* utvrđeno je da ispitivane linije pokazuju različitu genotipsku specifičnost u primenjenim testovima. Dobijeni rezultati su detaljno diskutovani u kontekstu istraživanja reproduktivne biologije ZP linija kukuruza, i imaju za cilj stvaranje preduslova za uspešno rukovodjenje i usmeravanje tog procesa u praksi - semenarskoj proizvodnji u odredjenim agroekoloških uslovima.

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