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VIABLE SUSPENSIONS OF MAIZE (*Zea mays* L.) POLLEN WITH EXOGENOUS DNA

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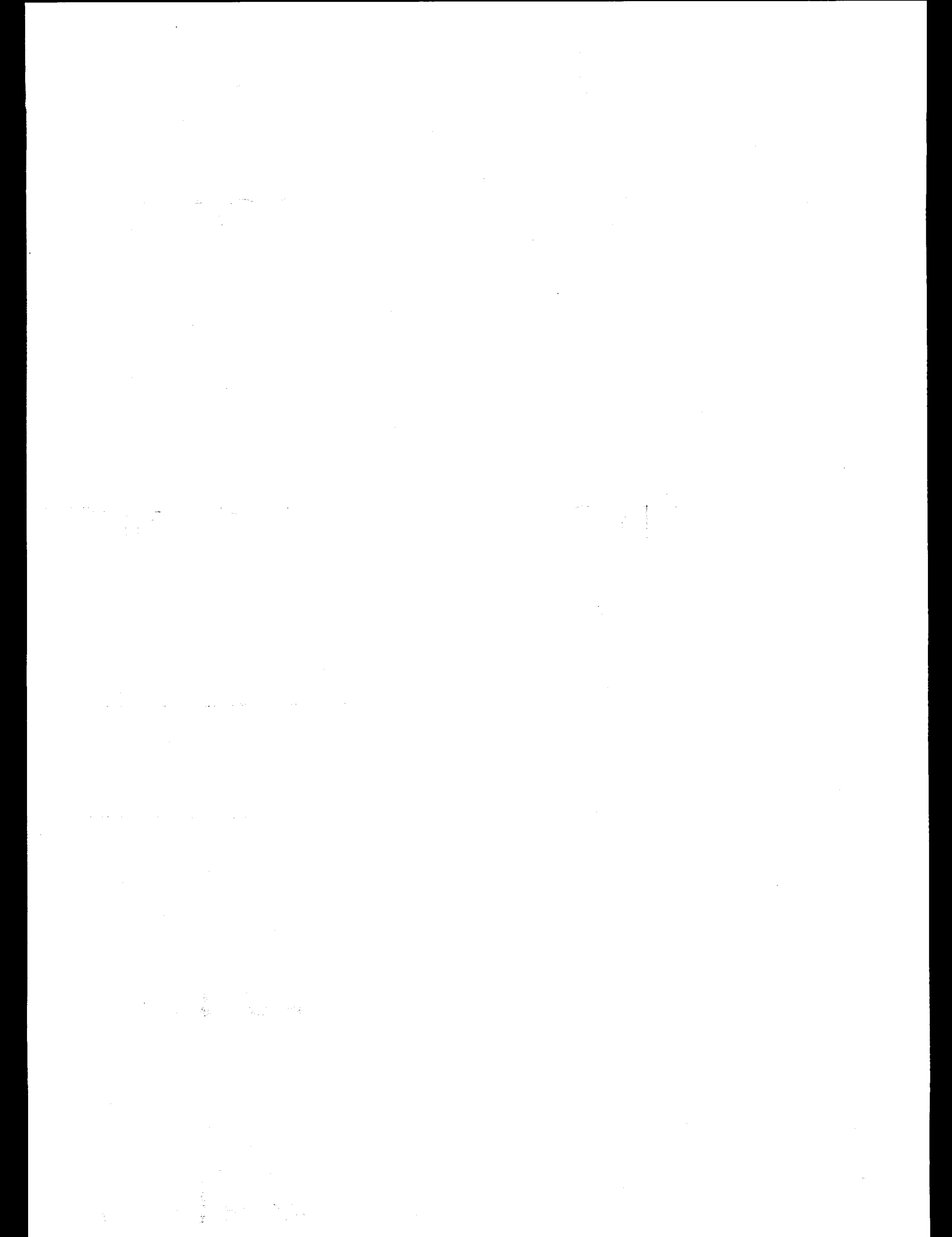
ABSTRACT

A viable suspension of maize pollen in aqueous medium containing exogenous DNA would be a suitable tool in attempting maize genetic transformation via pollen grains by different techniques. In this work the effects of addition of DNA to hypertonic aqueous media able to preserve maize pollen viability were investigated. An almost total loss of viability was found when pollen was incubated with native DNA in water or sucrose medium due to the immediate sticking of DNA on the pollen wall. Calcium in the incubation medium avoided DNA sticking preserving pollen fertilization ability. Pre-washing of pollen in hypertonic sucrose solution was proved to remove DNA binding components from the pollen wall. PEG 20%, that is known to inhibit pollen and silk nucleases, was also used instead of sucrose, without any reduction in the seed-set yields.

Key words: Maize, Pollen, Genetic transformation

RIASSUNTO

Una sospensione vitale di polline di mais in un mezzo acquoso contenente DNA esogeno risulterebbe un utile strumento per diverse tecniche di trasformazione genetica mediante polline. In questo lavoro sono stati studiati gli effetti dell'aggiunta di DNA ad un mezzo acquoso ipertonico in grado di mantenere la capacità di fertilizzazione del polline di mais. Quando il polline è stato incubato con DNA nativo in acqua o in soluzione di saccarosio, si è rilevata una pressochè totale perdita di vitalità dovuta ad un immediato attacco del DNA sulla parete del polline. Il calcio nel mezzo di incubazione, legandosi al DNA, ne ha evitato l'attacco alla parete del polline preservando le capacità di fertilizzazione di quest'ultimo. Il lavaggio del polline a freddo in una soluzione ipertonica di saccarosio, prima dell'incubazione con DNA, ha rimosso dalla parete del polline i componenti che legano il DNA. Poichè il PEG 20% è noto inibire le nucleasi del polline e delle setole di mais, la sostituzione del saccarosio nel mezzo di storage con PEG è stata sperimentata con successo.



1 Introduction

An ice-cold hypertonic sucrose medium has been found to preserve maize pollen *in vitro* germination /2/ and allow for maize pollen manipulation in reduced nuclease activity /3/. A different hypertonic aqueous medium, osmotically balanced on the sucrose and calcium contents, has been proved to preserve maize pollen fertilization ability /1,4/.

In this work no attempts were made to induce genetic transformation, but several aqueous media were used to suspend maize pollen together with exogenous DNA and pollen viability was tested by both *in vitro* germination and *in vivo* fertilization.

2 Materials and Methods

a) Genomic DNA preparation

An anthocyanin inhibited inbred line (A1,A2,C1,C2,R), gift from Istituto Sperimentale per la Cerealicoltura, Bergamo, was used for genomic DNA preparation. The extraction was performed according to the following procedure /6,7/. Embryos from 100 gr of germinating seeds were homogenized in cold (-20 °C) ethylene glycol and the homogenate filtered on Miracloth. The filtrate nuclear fraction was centrifuged (20000xg, 10 min, 4 °C), the pellet was dissolved in 150 ml of an ice-cold lysis buffer (1 M NaCl, 0.1 M Na-glycine pH 9.5, 10 mM EDTA, 1% N-lauroyl-sarcosine, 200 µg/ml Proteinase K) and incubated at 37 °C for 15-20 hours. The suspension was then centrifuged (35000xg, 20 min) and supernatant was gently shaken with 1 volume of phenol-chlorophorm 1:1, saturated with 10 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA pH 8 buffer. The aqueous phase, recovered by centrifugation (1000xg, 15 min), was further deproteinized by gentle shaking with 1 volume of chlorophorm-isoamyl alcohol 99:1, until clarification. No attempts were made to eliminate RNA. DNA was then precipitated by adding 2.5 volumes of ethanol 95%, spooled on glass rods and stored in isopropyl alcohol.

To perform pollen incubation experiments, DNA was dissolved in sterile distilled water. Absorbance at 260 nm and 280 nm and gel electrophoresis were determined to establish DNA purity, concentration and molecular weight. Only DNA preparations with (A₂₆₀/A₂₈₀) > 2 and molecular weight >10 kb were used. For PEG-calcium pollination experiments the pDPG232 plasmid carrying cDNA of the Sn maize gene (gift from

Dipartimento di Genetica e di Biologia dei microrganismi, Università di Milano) was used.

b) Plant growth conditions, pollen-DNA incubation, pollination

About 200 plants of a genetic line, obtained as F3 of a single cross between the inbred line A188 and an anthocyanin pigmented inbred line (gift from Limagrain Genetics, Cremona) were used during this work. At the flowering, ears and tassels were bagged and daily ground irrigation was applied. Date of silk emergence was noted on ear bag. Pollen was collected in the morning (h 9.00-10.00), filtered on a large size nylon sieve and checked for water content.

Pollen containing 50-60% of water was used. This pollen could be stored at least 10-12 hours in an ice-cold aqueous medium containing 0.6 M sucrose, 3 gr/l $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Ca 10x) /1,4/. Therefore, such a medium was used at room temperature to identify a proper DNA concentration which pollen would be incubated with. The medium previously sampled in 1 ml eppendorf tubes was complemented with increasing amounts of a high concentration DNA solution in water (e.g. 0, 10, 30, 50, 100 $\mu\text{g/ml}$). About 1/10 volume of freshly collected pollen was suspended in each tube and left to settle down. The DNA concentration just preceding the one producing non-settling suspensions of pollen (see later and Fig. 2) was used for experiments of DNA incubation. To do this, pollen was added to ice-cold storage medium with DNA (5 ml medium, 0.5-1 ml fresh pollen), vigorously shaken and maintained in ground ice.

For PEG-calcium experiments, an ice-cold solution of 20% PEG₄₀₀₀ was complemented with 6 gr/l $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Ca 20x), required for pollen stability /4/, and with 50 $\mu\text{g/ml}$ of pDPG232 plasmid. Pollen was added to such medium and stored in ground ice.

During incubation, pollen viability was repeatedly checked by liquid *in vitro* germination /1,4/: to promote germination 5-10 μl of pollen suspension were mixed with 95-90 μl of an ice-cold recovering medium, containing 0.5 M sucrose, 100 mg/l H_3BO_3 , spread on a glass slide for microscope observation and kept under high humidity conditions, at room temperature. At the time of pollination (after 5-10 hours of storage) the pollen suspension was reduced to a paste by filtering it on a two-layer nylon(70 μm)-paper filter. The pollen paste was placed on uncut, four days emerged, ear silks by a spatule and distributed by hand. The pollinated ear was then covered by a paper bag. One month after pollination seed-sets were assessed.

3 Results and Discussion

Following previous evidence on the osmotic role of all the solutes in aqueous medium /1/, the osmotic effect of DNA on maize pollen was initially investigated. Various amounts of genomic DNA (10-50 $\mu\text{g/ml}$, depending on DNA preparation) in distilled water allowed to obtain very stable (non-bursting, non-germinating) maize pollen suspensions, with a characteristic yellow appearance (Fig. 1). However, neither germination nor fertilization were ever observed with this pollen. Under microscope observation this pollen incubated with native DNA looked unable to complete hydration and, if repeatedly washed and transferred in distilled water, did even not recover the original aptitude to burst. Rather than a direct osmotic effect of DNA it could be supposed that high molecular weight DNA tightly sticks to the pollen grain and modifies its osmotic properties.

When genomic DNA was added to a hypertonic aqueous medium containing 0.6 M sucrose, 3 gr/l $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and then pollen was suspended in it, the physical aspect of the suspensions with increasing DNA concentration exhibited a remarkable discontinuity (Fig. 2). Increase of DNA increased the medium turbidity while pollen settled to the bottom of the tube in a few minutes after shaking. Nevertheless, for a particular DNA concentration range (depending on DNA preparation) pollen could stay suspended for several hours, before settling down. A further increase of the DNA concentration caused a completely inverted behaviour: pollen settled to the bottom in a few minutes leaving a very clear supernatant medium.

It is known that native DNA can be precipitated with calcium due to its numerous phosphate groups. Nevertheless the present experimental evidence has been useful to understand DNA-pollen interactions. The increasing turbidity in the medium until the condition of non-settling pollen can be explained by a diffuse Ca-DNA precipitate. The presence of calcium in the medium was an essential condition to keep DNA away from the pollen wall. When DNA was increased to exhaust the available calcium in the medium, the strong DNA affinity for the pollen wall reappeared with effects identical to the mentioned ones (quick pollen settling, yellow appearance, loss of bursting aptitude, loss of germination and fertilization abilities). These findings seem to support the hypothesis that the same phosphate bonds of DNA are involved both with calcium and with some unknown components of the pollen wall (e.g. calcium itself, basic proteins, etc.) /5/. It has been verified that small concentrations of Na_3PO_4 in distilled water

produced on a pollen suspension the same apparent effects noticed for pollen suspensions with native DNA (see Fig.1(f)).

Pollen of all the suspensions reported in Fig. 2, but the last one, when stored at 0 °C could then be induced to germinate by adding 9 volumes of an ice-cold recovering solution 0.5 M sucrose, 100 mg/l H_3BO_3 . In addition, the dispersion condition of non-settling pollen was particularly favourable for storage: pollen stored at 0 °C in this condition exhibited high *in vitro* germination rates after up to 3 days of storage. As to the failure of DNA coated pollen to germinate, at present it is impossible to discriminate between a deficiency of the available calcium or a modification of the osmotic properties of the pollen wall.

When pollen incubated several hours with Ca-DNA was used for *in vivo* pollination, a further restriction on DNA concentration has been evidenced. Pollen was filtered to a paste, as previously described [1,4], and applied to the ear silks by hand: an increasing difficulty in pollen distribution and reduction in pollen sticking to the silk was noted for pollen incubated with increasing DNA concentrations, resulting in less abundant seed-sets when pollen in the non-settling condition (optimum for storage) was used. An operative working DNA concentration for *in vivo* pollination was identified with the one just preceding the non-settling pollen condition (10-50 µg/ml depending on DNA preparation).

On the basis of the experimental evidence discussed above, it seems possible to conclude that native DNA tends to tightly stick to the pollen wall causing a drastical reduction of its fertilization ability. Calcium ions must be added to the medium for the dual purpose of salting DNA phosphate bonds (thus keeping DNA away from the pollen wall) and of supporting pollen for germination.

A last consideration could be raised about a possible reduction of the integration ability of exogenous Ca-DNA compared to native DNA. Therefore, the possibility to safely remove DNA binding components from the pollen wall was also investigated. After a few washings in an ice-cold hypertonic sucrose solution (1 M), pollen was suspended in 1 M sucrose containing DNA, at 0 °C. Then the medium was removed, pollen re-washed in 1 M sucrose and finally stored in 1 M sucrose, 3 gr/l $Ca(NO_3)_2 \cdot 4H_2O$ at 0 °C. High germination percentages have been obtained from this pollen, when recovered by 9 volumes of 0.5 M sucrose, 100 mg/l H_3BO_3 (Fig. 3). These results support the possibility of incubating pollen with native DNA, after washing DNA binding components from the pollen wall.

Since PEG 20% has been proved to inhibit pollen and silk nucleases /8/, the effect of replacement of sucrose with PEG on the obtainable seed-sets was investigated. In such cases 50 µg/ml of pDPG232 plasmid DNA have been added to PEG₄₀₀₀ 20%, Ca_{20x}, without any apparent DNA precipitation. Pollen stored several hours in this medium, filtered into a paste and distributed on ear silks, produced seed-sets such as those reported in Fig. 4. This medium could then be used to attempt maize genetic transformation via pollen grains.

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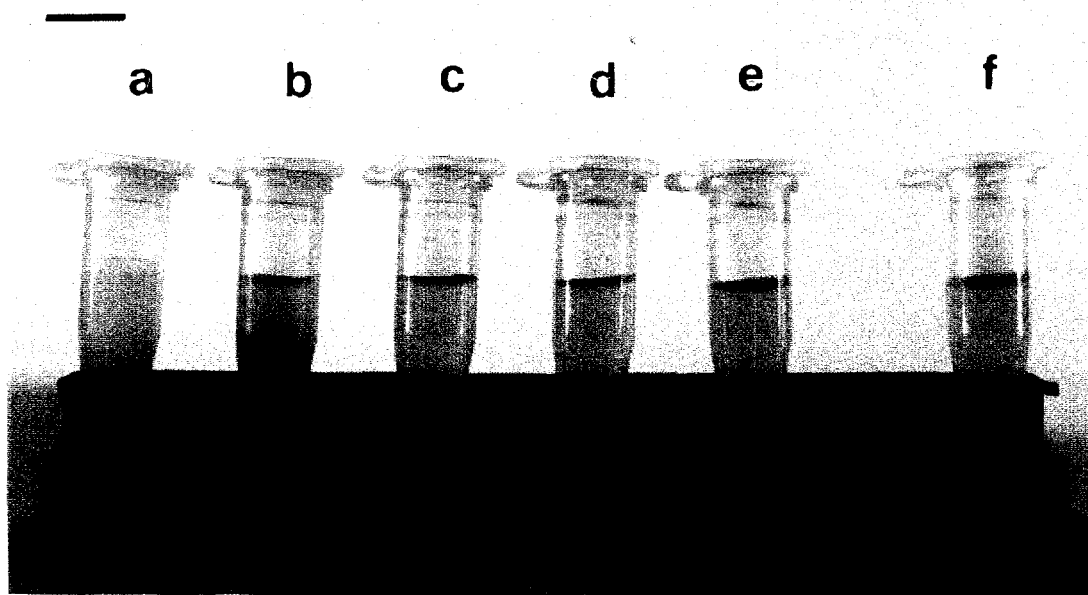


Fig. 1 - Maize pollen suspended with different concentrations of genomic DNA in water (bar=1cm): (a) 0 $\mu\text{g/ml}$, (b) 10 $\mu\text{g/ml}$, (c) 20 $\mu\text{g/ml}$, (d) 30 $\mu\text{g/ml}$, (e) 50 $\mu\text{g/ml}$; (f) maize pollen suspended in 5 mM Na_3PO_4 .

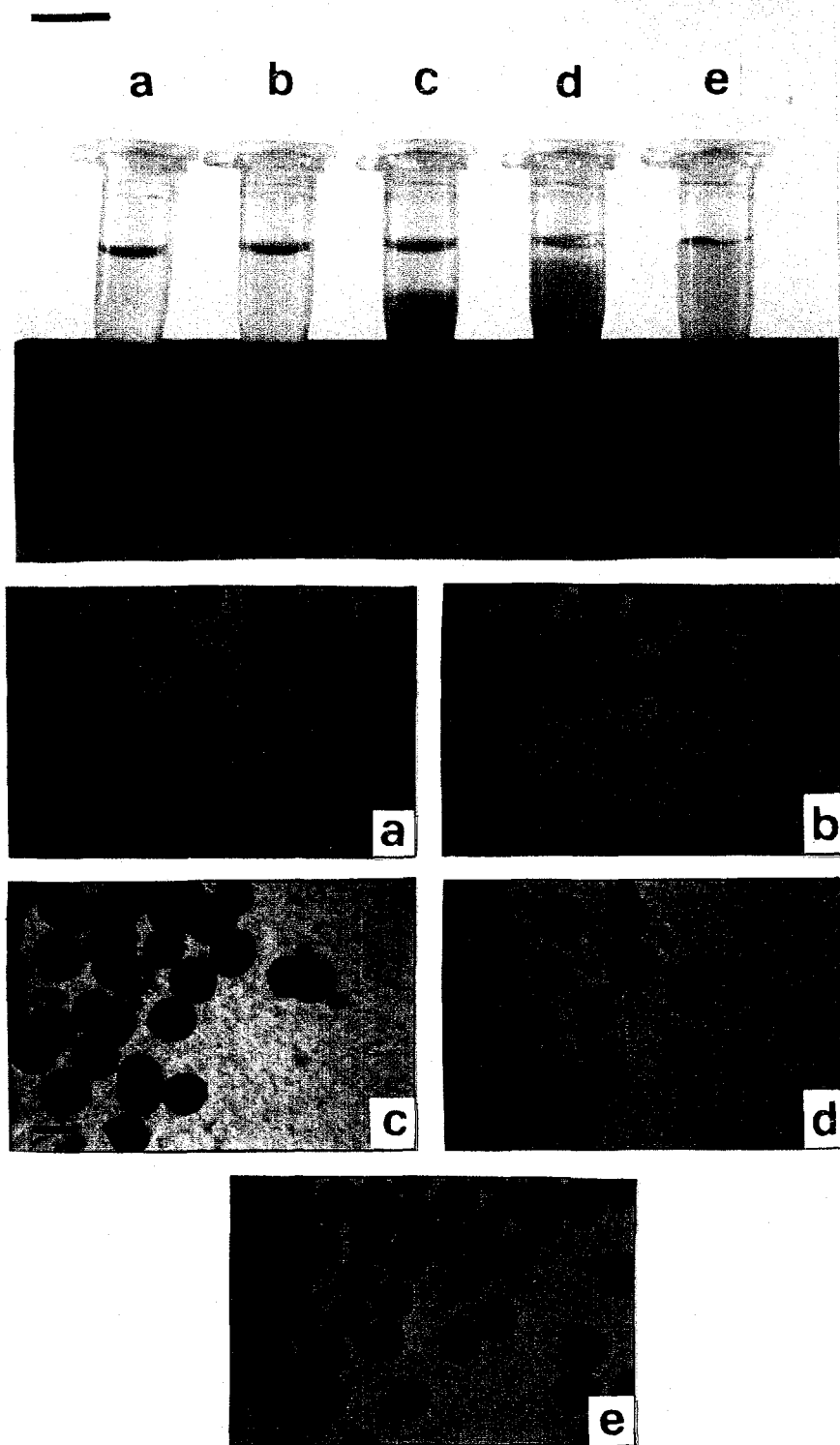


Fig. 2 - Macroscopic (bar=1cm) and microscopic (bar=100 μ m) appearance of maize pollen suspensions in 0.6 M sucrose, 3 gr/l $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, containing increasing concentrations of genomic DNA: (a) 0 $\mu\text{g}/\text{ml}$, (b) 10 $\mu\text{g}/\text{ml}$, (c) 30 $\mu\text{g}/\text{ml}$, (d) 50 $\mu\text{g}/\text{ml}$, (e) 100 $\mu\text{g}/\text{ml}$.

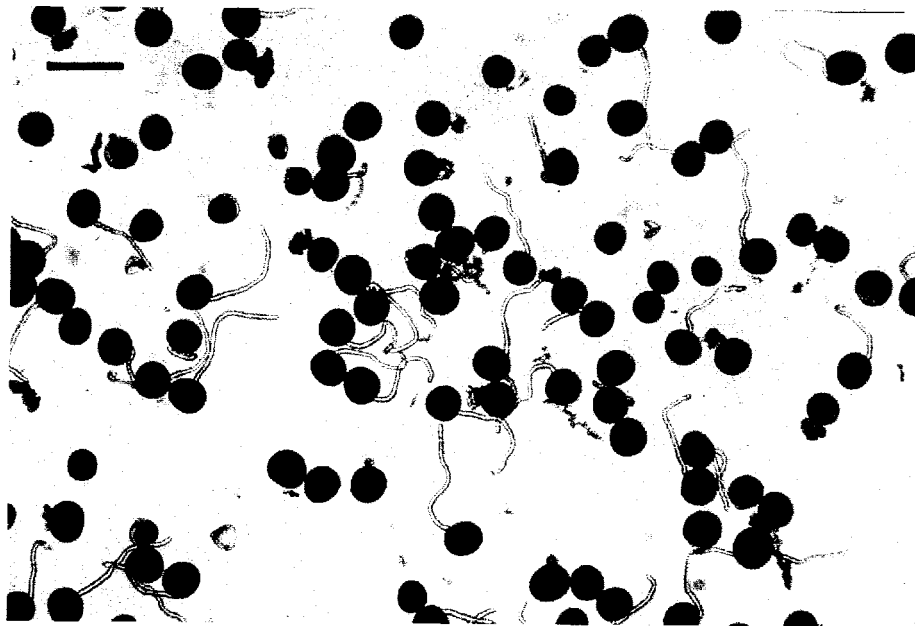


Fig. 3 - *In vitro* germination of maize pollen after removing DNA binding components from the pollen wall and incubating with genomic DNA (bar=200 μ m). Pollen was previously washed (three times) in ice-cold 1 M sucrose and then incubated in 1 M sucrose, 50 μ g/ml DNA, at 0 °C. After 1 hour, pollen was re-washed in ice-cold 1 M sucrose and then stored in 1 M sucrose, 3 g/l $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ at 0 °C. For *in vitro* germination 10 μ l of pollen suspension were mixed with 90 μ l of an ice-cold medium containing 0.5 M sucrose, 100 mg/l H_3BO_3 and spread on a microscope glass slide, kept in high humidity conditions, at room temperature.

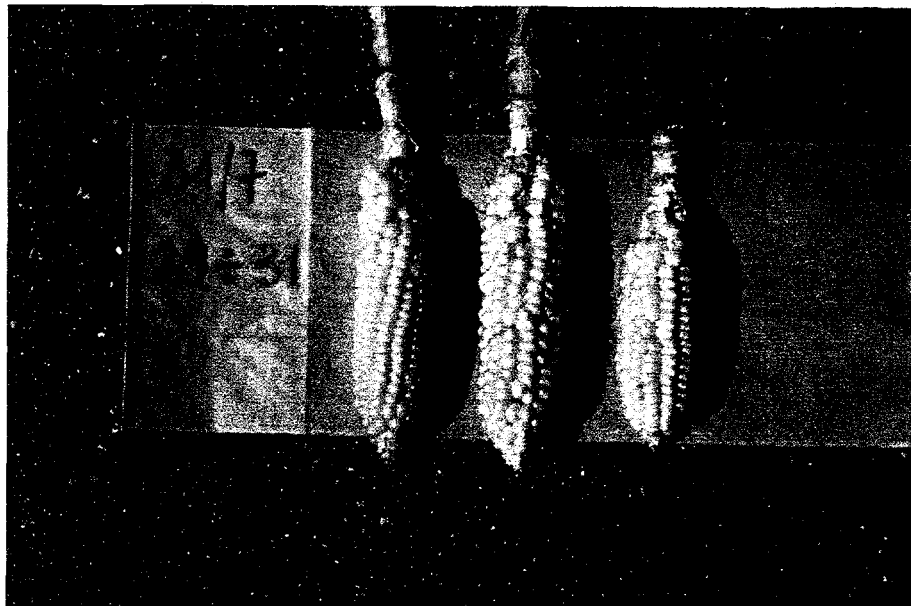


Fig. 4 - Maize seed-sets obtained by *in vivo* pollination with pollen previously incubated in PEG-calcium medium together with the pDPG232 plasmid (bar=5cm). Pollen was harvested at 10.00 a.m. (water content 59%) and incubated in an ice-cold medium (5 ml medium/0.5 ml pollen) containing 20% PEG₄₀₀₀, 6 gr/l Ca(NO₃)₂·4H₂O and 50 µg/ml of pDPG232 plasmid, at 0 °C. At the time of pollination (18.00 p.m.) one sample was filtered to a paste, immediately applied to four days emerged ear silks and distributed by hand.

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