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BRIEF COMMUNICATION

# Impairment in reproductive development is a major factor limiting yield of black gram under zinc deficiency

N. PANDEY*, G.C. PATHAK and C.P. SHARMA

*Botany Department, University of Lucknow, Lucknow-226007, India*

## Abstract

Black gram [*Vigna mungo* (L.) Hepper] cv. IPU 94 plants grown in sand culture with deficient zinc (0.1 μM Zn) nutrition and those deprived of normal (1 μM) Zn supply at the initiation of flowering, showed decrease in dry matter production and especially seed yield. These plants showed a decrease in the size of anthers and stigmatic heads, pollen producing capacity of the anthers and stigmatic exudations. Zn deficiency caused structural alterations in exine and retarded germination of pollen grains and tube growth. The pollen extracts and stigmatic exudates of the Zn-deficient plants showed increase in activity of acid phosphatase isoforms and inhibition of esterase isoforms. Zn deficiency led to decrease in number of pods, seeds per pod and seed mass, altered seed coat topography and reduced seeds germinability. Low seed yield under Zn deficiency is attributed to a role of Zn in pollen function, as also in pollen-pistil interaction conducive to fertilization and development of seeds.

*Additional key words*: pollen grains, reproduction, stigma exudates, *Vigna mungo*.

Zinc is an essential plant nutrient that functions in diverse metabolic, regulatory and developmental processes (Sharma 2006, Shi and Cai 2009). It has also been shown to be involved in defense against toxic effects of reactive oxygen species (Cakmak 2000). There is increasing concern about Zn nutrition of plants because its deficiency is widely spread in the arid and semi-arid regions of the world (Takkar and Walker 1993). Grain yield of crops may be decreased substantially even when Zn deficiency effect on shoot biomass yield is marginal (Brennan 1992, Pandey and Sharma 1998), which indicates a role of Zn in reproductive development. In the form of zinc finger proteins, Zn has also been shown to be involved in expression of genes related to microsporogenesis (Kobayashi *et al*. 1998, Takatsuji 1999) and megagametogenesis (Grossniklaus *et al*. 1998, Brive *et al*. 2001).

Evidence of a role of Zn in plant reproduction is presently confined to pollen fertility (Sharma *et al*. 1987, 1990, Pandey *et al*. 1995, 2000, 2006), even though there are several other stages in reproductive development having a direct bearing on seed yield. Little attention has been paid on study of Zn nutrition on pollen-pistil interaction that is crucial to fertilization (Dickenson and Elleman 1994, Taylor and Hepler 1997, Hiscock 2004). Fertilization could also be regulated by specific enzymes like proteases (Radlowski *et al*. 1996), acid phosphatases (Roggen and Stanley 1969), esterases (Dafni and Maues 1998) and cutinases (Hiscock *et al*. 1994, Edlund *et al*. 2004). Growth of embryo and initial stages of seed maturation are known to be influenced by nutrition of the mother plants (Weber *et al.* 2005). These possibilities have, however, not been investigated in response of Zn nutrition of the mother plants. The present study is a step in this direction.

Black gram [*Vigna mungo* (L.) Hepper] cv. IPU 94 was grown in sand culture (Sharma 1996). The nutrient solution contained macronutrients [mM] Ca $(NO_3)_2$ (4), $KNO_3$ (4), $MgSO_4$ (2), $NaH_2PO_4$ (1.33), $H_3BO_3$ (0.33), Fe-$K_2$ EDTA (0.1), NaCl (0.1), micronutrients [μM] $MnSO_4$ (10), $CuSO_4$ (1.0), $Na_2MoO_4$ (0.1), $CoSO_4$ (0.1) and $NiSO_4$ (0.1) and Zn in the form of $ZnSO_4$ at two concentrations - 1.0 μM (Zn-sufficient) or 0.1 μM (Zn-deficient). With a view to exploring the role of Zn in

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*Abbreviations*: PPC - pollen producing capacity; SEM - scanning electron microscopy.
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* Corresponding author; fax: (+91) 05222740540; e-mail: nalini_pandey@rediffmail.com

reproductive development, at the initiation of flowering (floral bud initiation; day 50), pots with Zn-sufficient and Zn-deficient plants were each separated in two lots (of eight pots each). Zinc was withheld from one of the two lots of the former and 1 μM Zn was supplied to one lot of the latter. Thus four Zn treatments were used: Zn-sufficient (ZnS), Zn-deficient (ZnD), Zn-sufficient turned to Zn-deficient (ZnS→D) and Zn-deficient turned to Zn-sufficient (ZnD→S). During the course of the experiment (upto harvest, day 100), the photoperiod was 12 h with maximum irradiance (PPFD) at 12:00 ranging between 1 000 and 1 200 μmol $m^{-2}$ $s^{-1}$. Temperature ranged between 35 to 40 °C (max) and 28 to 34 °C (min). The RH around 09:30 ranged from 78 to 98 %. Dry matter, leaf tissue concentration of Zn and seed yield was quantified at harvest (day 100), and the morphological changes in pollen exoskeleton, pollen receptive surface of the stigma and seed coat topography at day 65. At this stage, the isoenzymes of acid phosphatase and esterase were also visualized on stigmatic surface and extracts of pollen grains and stigma by activity staining after PAGE separation.

Leaf tissue concentration of Zn was determined by atomic absorption spectrophotometer (*Perkin Elmer Analyst 300*, The Netherlands) after wet acid digestion ($HNO_3$:$HClO_4$; 10:1) of oven dried (80 °C) samples. The size of 20 mature, randomly selected flowers (length from the base of the calyx to the tip of the standard petal) was measured. Anther size was approximated by measuring the length and breadth of the anther lobes. For light and scanning electron microscopy (SEM) fresh flowers were collected between 07:00 to 09:00 and fixed in formaldehyde:acetic acid:ethyl alcohol (FAA). For SEM, the flowers were removed from FAA, refixed overnight in 1.5 % glutaraldehyde in 0.05 M phosphate buffer pH 7.2, washed in phosphate buffer and postfixed in 1 % (m/v) osmium tetroxide in the same buffer for 2 h at 4 °C. Acetolysis of pollen grains was carried out in freshly prepared solution of acetic anhydride and sulphuric acid (9:1) at 70 - 80 °C (Erdtman 1986). The dehydrated specimens were mounted on brass stubs, sputter coated with gold and viewed in *Philips* (The Netherlands) SEM at an accelerating voltage of 15 kV. The pollen producing capacity (PPC) was counted in a set of 5 flowers and denotes the number of pollen grains dispersed from an anther (Shivanna and Rangaswamy 1992). Size of anthers and pollen grains and thickness of exine were measured in a set of five flowers after mounting the preparations in glycerine jelly. Pollen viability was determined by measuring *in vitro* germination percentage of pollen grains in a medium containing 15 % sucrose, 0.01 % boric acid, 0.03 % calcium nitrate, 0.02 % magnesium sulphate and 0.01% potassium nitrate (Brewbaker and Kwack 1963), maintained at 30 °C in flowers drawn from ten different plants between 08:00 and 09:00. Pollen grains producing pollen tubes equal to or more than the pollen diameter were treated as germinated. Scoring was based on 10 sets of 20 pollen grains for each treatment using a *Nikon E-400* (Japan) microscope. For visualizing pollen germination and pollen tube growth on the stigmas, pistils of flowers fixed for pollen grain morphology were incubated at 45 °C for 24 h in a staining mixture containing 1 % of each malachite green, acid fuchsin, aniline blue and orange G in lactic acid (Alexander 1987). The stained pistils were then transferred to clearing and softening mixture containing lactic acid, phenol and orange G for 48 h at 45 °C. The stigmas were washed in lactic acid, mounted in a solution of lactic acid and glycerol (1:1) and observed under the microscope.

Assay for acid phosphatase (EC 3.1.3.2) activity was made in crude extracts of pollen grains in distilled water as described earlier (Sharma *et al*. 1987) and expressed in terms of μg Pi liberated per mg protein, the later being determined by the method of Bradford (1976). The assay system contained 0.05 M acetate buffer pH 5.0 and 0.01 M Na β-glycerophosphate. The reaction was stopped by adding 10 % trichoracetic acid (TCA). Acid phosphatase and esterase isoenzymes in the extracts of pollen grains and the stigma exudates of Zn-sufficient and Zn-deficient plants were separated on 10 % native PAGE and visualized by the method of Brewbaker *et al.* (1968). The gels were stained for acid phosphatase in a solution containing 0.1 M $MgCl_2$, 1 % α-naphthyl acid phosphate and *Fast Garnet GBC* salt in 0.2 M acetate buffer, pH 5.0, and for esterase in a solution containing 1 % α-naphthyl-acetate and *Fast Blue RR* in 0.3 M phosphate buffer (pH 7.2). All stains for the reaction mixture were obtained from *Sigma* (St. Louis, USA). Seed yield was determined in all the four treatments after harvest (day 100) and SEM examination for surface morphology of the seeds was made in the region adjacent to the hilum. The mature seeds were tested for viability (% germination) by placing them on moist filter paper in Petri dishes. Each treatment had three replicates and the expe-rimental data were analyzed statistically using *ANOVA*.

In plants raised with 0.1 μM Zn, growth of internodes, branching and leaf size were restricted and young trifoliates showed interveinal chlorosis. Flowering was delayed by 7 or 8 d, the number of floral buds and size of flowers was significantly reduced (Table 1) and a number of floral buds failed to open. Withholding Zn supply at initiation of flowering (ZnS→D) made little difference to flower size but supply of sufficient Zn to the Zn-deficient plants (ZnD→S) minimized the Zn-deficiency effects (Table 1). Zinc effect on plant biomass followed a similar trend (Table 1) but decrease in seed yield due to limitation in Zn concentration far exceeded (84 %) that in shoot dry matter production (43 %), which suggest a higher requirement of Zn for reproductive yield than for vegetative growth.

The ZnD plants showed a significant reduction in anther size and PPC (Table 1). In an earlier study with maize (Sharma *et a*l. 1987), it was observed that instead of developing into sporogenous tissue, the anthers of the Zn-deficient plants developed into vascular tissue. Decrease in PPC could also result due to Zn-deficiency

Table 1. Effect of Zn nutrition of black gram on leaf tissue Zn content, number and size of flowers, anther size, pollen characteristics, growth parameters and seeds characteristics. ZnS - Zn-sufficient, ZnD - Zn-deficient; ZnS→D - Zn supply withheld from ZnS at initiation of flowering, ZnD→S - Zn was supplied to ZnD at initiation of flowering. Means ± SE, $n = 3$.

| Parameter | ZnS | ZnD | ZnS→D | ZnD→S | $LSD_{0.05}$ |
|---|---|---|---|---|---|
| Tissue Zn content [μg $g^{-1}$(d.m.)] | 41.7 ± 0.67 | 13.1 ± 0.14 | 32.9 ± 0.54 | 26.2 ± 0.38 | 4.6 |
| Flower number [$plant^{-1}$] | 55.0 ± 0.45 | 42.0 ± 0.78 | 51.0 ± 0.65 | 46.0 ± 0.36 | 4.0 |
| Flower size [cm] | 1.5 ± 0.03 | 1.2 ± 0.02 | 1.4 ± 0.37 | 1.3 ± 0.01 | 0.12 |
| Anther size [μm] | 795.0 ± 10.0 | 579.0 ± 5.80 | 718.0 ± 12.7 | 692.0 ± 9.80 | 19.0 |
| Pollen grains number [$anther^{-1}$] | 579.0 ± 5.70 | 289.0 ± 3.90 | 365.0 ± 6.40 | 342.0 ± 4.90 | 23.0 |
| Pollen size [μm] | 90.2 ± 3.90 | 82.1 ± 6.70 | 88.5 ± 4.80 | 86.3 ± 6.30 | 2.7 |
| Exine thickness [μm] | 3.2 ± 0.13 | 4.1 ± 0.37 | 3.4 ± 0.24 | 3.4 ± 0.23 | 1.1 |
| Pollen germination [%] | 76.0 ± 0.96 | 42.0 ± 0.56 | 56.0 ± 0.78 | 51.1 ± 0.98 | 7.0 |
| Acid phosphatase [μg(Pi) $mg^{-1}$(protein)] | 60.0 ± 0.65 | 121.0 ± 0.97 | 113.0 ± 2.06 | 105.0 ± 3.47 | 5.0 |
| Pods number [$plant^{-1}$] | 30.0 ± 0.34 | 25.0 ± 0.65 | 28.0 ± 0.78 | 26.0 ± 0.67 | 3.0 |
| Shoot dry mass [g $plant^{-1}$] | 7.2 ± 0.48 | 4.1 ± 0.09 | 5.7 ± 0.08 | 5.4 ± 0.17 | 1.4 |
| Pod dry mass [g $plant^{-1}$] | 5.6 ± 0.36 | 2.4 ± 0.17 | 3.9 ± 0.21 | 3.3 ± 0.27 | 1.2 |
| Total yield [g $plant^{-1}$] | 12.9 ± 0.68 | 6.5 ± 0.78 | 9.6 ± 0.27 | 8.7 ± 0.56 | 2.3 |
| Seed number [$plant^{-1}$] | 120.0 ± 0.36 | 52.0 ± 0.43 | 98.0 ± 1.89 | 70.0 ± 2.06 | 8.0 |
| Seed dry mass [g $plant^{-1}$] | 4.5 ± 0.03 | 0.7 ± 0.02 | 2.6 ± 0.35 | 1.4 ± 0.37 | 0.11 |
| One seed mass [mg] | 37.0 ± 0.59 | 15.2 ± 0.37 | 26.7 ± 0.42 | 19.2 ± 0.36 | 0.05 |
| Seed germination [%] | 80.0 ± 1.09 | 50.0 ± 0.69 | 71.0 ± 0.79 | 66.0 ± 0.96 | 4.5 |

induced changes in structure and function of tapetum by virtue of a role of Zn as a structural motif of the Zn finger proteins and their role in anther development (Kobayashi *et al*. 1998, Takatsuji 1999). Takahashi *et al*. (2003) have attributed aberrations in floral parts including the anthers of Zn-deficient plants to impairments in nicotianamide (NA) dependent chelation and transport of Zn to floral parts. They observed that transfer of *naat* gene from tobacco mutant lacking in NA synthesis to transgenic plants of barley over-expressing NA synthesis gene (*HvNAS1*) enhanced Zn transport to the reproductive parts and reversed the floral aberrations in the former. We observed a decrease in size of the pollen grains, thickening of exine and changes in exine ornamentation of ZnD pollen grains (Table 1; Fig. 1*B,D*). Black gram has three zonoporate pollen grains with reticulate ornamentations. While the exine of ZnS pollen grains showed penta- to hexagonal reticulations with uniform muri (Fig. 1*A,C*), that of ZnD showed incomplete reticulations with sinuos muri (Fig. 1*B,D*). The muri of ZnD pollen grain appeared lobed and covered with waxy depositions, with the lumen showing prominent raised baculae (Fig. 1*D*). Similar, but less prominent, changes in exine topography were produced in ZnS→D pollen grains. The ZnD pollen grains failed to stain with acetocarmine (Fig. 2*A,B)* and showed a significant decrease in *in vitro* germinability (Fig. 2*C,D)* suggesting loss of viability. Pollen tube growth was also retarded and many of them showed bursting of the growing tip (Fig 2*D*). Not only *in vitro* germination but germination and pollen tube growth on the stigmatic surface was also limited by Zn deficiency (Fig 3*A,B*). Increases in thickening and changed ornamentations of exine seemed to be a factor determining their germination and pollen tube growth. Thickening of the exine and raised and wider muri with waxy deposition filling the cavities between the baculae could create a hydrophobic environment detrimental to pollen hydration which is a prerequisite for germination and growth of pollen tubes (Taylor and Hepler 1997, Hiscock 2004) and hence crucial for affecting fertilization. Franklin-Tong (1999) and Zinkl *et al*. (1999) have shown that alterations in pollen coat waxes and lipids may lead to loss of pollen fertility.

Scanning electron microscopy of stigmas from flowers of ZnD plants showed a decrease in the pollen receptive area and a persistent cuticle over the stigmatic surface. This prevented the secretion of stigmatic exudates as a consequence of which fewer pollen grains adhered to the ZnD stigmas and their germination and pollen tube growth was restricted (Fig. 3*B*). Rupture of the cuticle over the stigmatic papillae is crucial to adhesion of pollen grains on the stigma (Heslop-Harrison 2000). It is suggested that the rupture of the stigmatic cuticle possibly involves the activities of certain enzymes such as cutinases (Hiscock *et al*. 1994, Edlund *et al.* 2004) and esterases (Dafni and Maues 1998, Hiscock *et al*. 2002). We found that Zn deficiency led to increase in activity of acid phosphatase both in stigmatic and pollen extracts. Four isoforms of acid phosphatase were expressed in the stigma exudates (Fig. 5*A*) and three in pollen extracts in ZnS plants (Fig. 5*B)* and all of these were expressed to a greater extent in ZnD plants (Fig. 5*A*,*B*). Enhancement of pollen acid phosphatase activity is reported to be inhibitory to pollen tube growth (Roggen and Stanley 1969). Opposite to the effect on acid phosphatases, six esterase isoforms were observed in the stigmatic extracts of ZnS plants but only four in ZnD

plants. (Fig. 5*C*). In pollen extracts also, as against three esterase isoforms in ZnS plants, only two were feebly expressed in ZnD plants (Fig. 5*D*). It is likely that observed changes in the activities of stigmatic and pollen esterase and acid phosphatase induced in response to Zn deficiency stands in way of a favorable pollen-pistil interaction and limits fertilization providing an explanation to decrease in the number of pods. However, these changes do not explain the poor development of seeds and changes in seed coat topography (Fig. 4*A,B*). The ZnS seeds had a rugate surface with prominent interrugosity connections and thin waxy depositions (Fig. 4*A*). In comparison, ZnD seeds had thick waxy depositions which masked the rugulate pattern and made the interrugosity connections appear thick and waxy (Fig. 4*B*). Zinc deficiency also reduced the germinability of seeds (Table 1). Increase in Zn supply from ZnD to ZnS at the initiation of flowering (ZnD→S) partially mitigated the Zn effects on the seeds and their germinability. These changes are suggestive of a role of Zn in seed development and maturation. Further work is required to substantiate this. It is however, known that the seed development in legumes is controlled by diverse maternal inputs (Weber *et a*l. 2005) and that in *Arabidopsis,* zinc finger transcription factors have a positive influence on seed germination (Liu *et al.* 2005). We concluded that loss of pollen function, impairment in fertilization and poor development of seed all contribute to poor seed yield of legumes grown on low Zn soils and that this can partly be compensated through Zn fertilization of crops at the onset of reproductive phase.

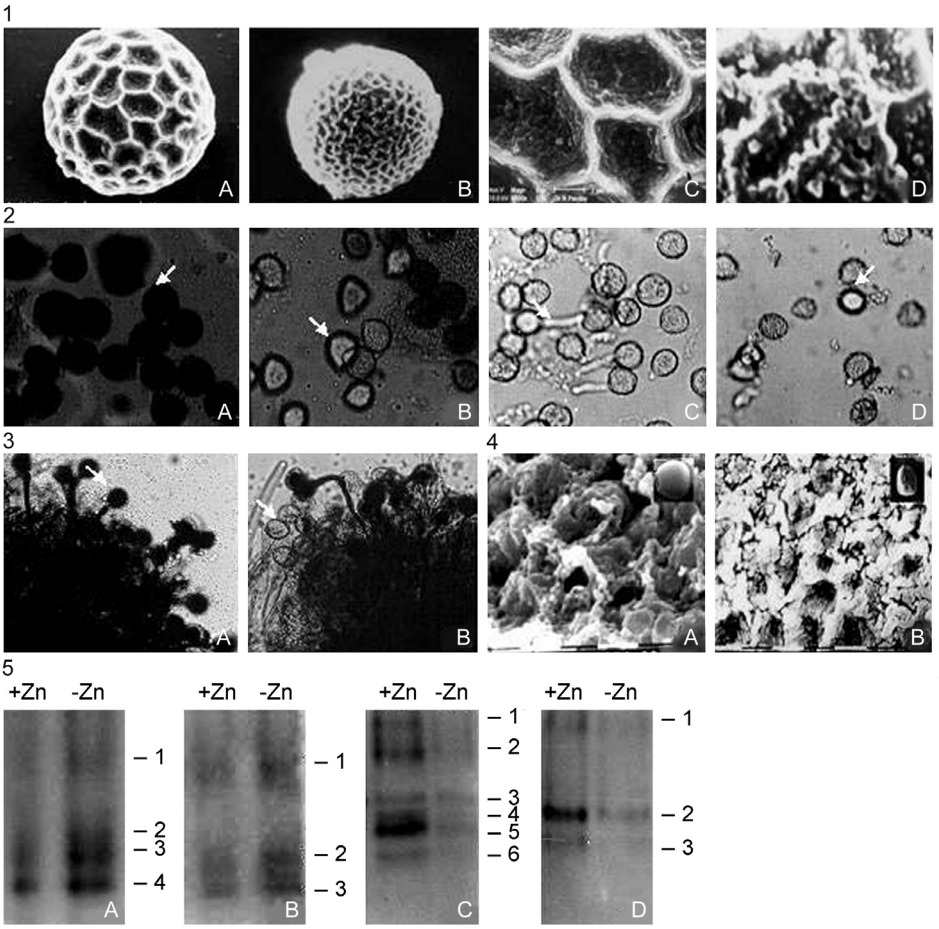


Fig. 1. Scanning electron microscopy (SEM) images of Zn-sufficient (*A*) and Zn-deficient (*B*) pollen grains. Pollen grains of *A* show uniform muri (*C*); those of *B* show sinuous lobed muri, with waxy depositions filling the cavity between baculae (*D*). *Bars* = 10 μM in *A,B*; 2 μM in *C,D.*

Fig. 2. Photomicrographs showing viable (*arrow*, *deeply stained*) pollen grains of Zn-sufficient (*A*) and non-viable pollen grains (*arrow*, *unstained*) of Zn-deficient (*B*) plants. *In vitro* germination of pollen grains showing well formed pollen tubes (*arrow*) of Zn-sufficient pollen (*C*) and burst tips (*arrow*) of Zn-deficient pollen tubes (*arrow*) (*D*).

Fig. 3. Stigma of Zn-sufficient flowers (*A*) showing many viable pollen grains with pollen tubes (*arrow*). Stigma of Zn-deficient flowers (*B*) showing fewer, non-viable (*arrow*, *unstained*) pollen grains..

Fig. 4. SEM images showing seed coat topography (whole seed inset) of ZnS (*A*) and ZnD (*B*) seeds. *Bars* = 10 μM.

Fig. 5. Activity staining of native gels showing Zn deficiency isoforms of acid phosphatase (*A, B*) and esterase (*C, D*) in stigma exudates (*A, C*) and pollen extracts (*B, D*).

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