Pollen Pistil Interaction in Inter-Specific Crosses of *Vigna* sp.

Krishnasamy Thiagu, Palaniappan Jayamani* and Nagasamy Nadarajan

Department of Pulses, Centre for plant Breeding and Genetics, Tamil Nadu Agricultural University Coimbatore-641 003, India

Received June 11, 2008; accepted August 11, 2008

**Summary**  Inter-specific hybridization is mainly used to introgress pest and disease resistant genes. *Vigna radiata* and *Vigna mungo* are widely cultivated and consumed in India. These crops are highly affected by Mungbean Yellow Mosaic Virus (MYMV) disease and bruchid (*Callasobruchus* spp.) pest, leading to heavy yield loss. *Vigna umbellata*, a divergent *Vigna* species is found to be resistant to both MYMV and bruchids. Utilization of this species to introgress the resistance gene is marginal because of their inherent low crossability with the cultivated *Vigna* species. In the present study, *V. umbellata* was used as pollen donors in hybridization with *V. radiata* and *V. mungo*. Low percentage of pod set observed in the cross *V. radiata*×*V. umbellata* (12.89%) and *V. mungo*×*V. umbellata* (5.56%) indicated the presence of reproductive barriers that render introgression difficult. Aim of this study was to observe pre-fertilization barriers operating in *V. radiata*×*V. umbellata* and *V. mungo*×*V. umbellata* crosses. *In vivo* pollen germination and growth of pollen tubes were studied in inter-specific crosses using fluorescence microscope. The pollen grain germination on stigmatic surface was normal. A very common pre-fertilization barrier of slow rate of pollen growth, in addition to structural abnormalities in stigmatic and stylar regions was observed in both the crosses. However, the level of incompatibility was high in *V. mungo*×*V. umbellata* cross than *V. radiata*×*V. umbellata*. Measures to overcome incompatibility barriers before fertilization in these crosses to produce inter-specific hybrids are discussed.

**Key words**  Inter-specific crosses, Pre-fertilization barriers, Fluorescence microscopy, Pollen tube growth, *Vigna* species

Pulse crops provide easily digestible, high quality protein for human consumption, green nutritious fodder for animal and enriches the soil through biological nitrogen fixation, hence are called as ‘poor man’s meat’ and ‘Unique Jewels’ of Indian crop husbandry (Swaminathan 1981). Legumes play a central role in low input production systems, particularly on small-scale farms (Graham and Vance 2003). Grain legumes rank third among the grain crops behind cereals and oilseeds in world production. The *Vigna radiata* (Greengram/Mungbean) and *Vigna mungo* (Blackgram/Urdbean) constitute an important dietary constituents for human which was grown in 3.21 and 3 m·ha area with a production of 0.98 and 1.37 mt, while its productivity is very low around 482 and 453 kg ha⁻¹, respectively (Ali and Kumar 2001). This may be due to dearth of high yielding varieties, lack of resistance to pest and disease and cultivation under rain-starved, unhealthy soil. In India, Mungbean Yellow Mosaic Virus (MYMV) and bruchids (*Callosobruchus* spp.) cause heavy damage to both the cultivated *Vigna* species and estimated the yield loss up to 85% (AVRDC 1998).

Wide hybridization has an important role in producing new association of plant characteristics that are outside the range of variability of the parental species (Asthana *et al.* 1994). The introgression of wild and related species of *Vigna* has also been reported from the 1970s (Ahuja and Singh 1977, Ahn and Hartmann 1978). Some of the germplasm accessions have the cultivated form of

---

* Corresponding author, e-mail: jayamani1108@gmail.com
Vigna species which are good plant type and resistant to pest and disease (Umamaheshwari 2002, Somta et al. 2007). The use of divergent species namely Vigna umbellata (Ricebean) is more desirable for introgression breeding due to no linkage drag of undesirable traits such as pod dehiscence and it is widely consumed by human than the other wild species (Watanasit and Pichitporn 1996). This species were found in Western Ghats, Eastern Ghats, and North Western Himalayas, which act as a potential source of resistance to disease such as MYMV and to insect pest such as bruchids (Monika et al. 2005, Somta et al. 2007).

The inter-specific cross hybridization and introgression are meant to tap genes of pest and disease for cultivars improvement. In previous attempts made between divergent relative species of V. umbellata germplasm accession and cultivated species of V. radiata and V. mungo have been less successful due to low crossability (Umamaheshwari 2002). An understanding of the reproductive isolation barrier systems that hamper wide hybridization and seed development would certainly assist in the production of inter-specific hybrids of Vigna. This could be due to both at pre-fertilization and post-fertilization levels. The mechanism of pre-fertilization barriers was observed in wide crosses namely inhibition of pollen germination, delayed pollen tube growth and structural aberrations of pollen tubes in Cotton (Gunasekaran 1997) and in Sesame (Rajeshwari and Ramasamy 2004, Ganesh Ram et al. 2006). Considering the above, the present investigations have been made with the objective to study the pollen pistil interaction of V. umbellata pollen tubes at various stages of pistils of V. radiata and V. mungo to determine whether reproductive isolation barriers are inhibiting the production of inter-specific hybrid derivatives.

### Materials and methods

Details of the plant materials used and its characteristic features are furnished in Table 1. The cultivated Vigna used were V. radiata cv CO 5 (2n=22) and V. mungo cv VBN1...
(2n=22), a well adapted high yielding cultivars and the cultivated divergent relative species used was *V. umbellata* cv RBL 1 (2n=22), a determinate, twiny and resistant to MYMV and bruchids. The parents used in this study were maintained as homozygous stocks by continuous selfing, in the Department of Pulses, Tamil Nadu Agricultural University, Coimbatore, India.

Crossoes were attempted between *V. radiata*×*V. umbellata* as well as *V. mungo*×*V. umbellata*. Artificial pollination was carried out following the method suggested by Bolling et al. (1961). For hybridization, the flowers in *V. radiata* and *V. mungo* (female parents) were emasculated in the evening, between 3–5 PM. The pistils were closed using petals after emasculation to avoid contamination. Next day morning between 7–9 AM, the pollen from *V. umbellata* (male parent) was transferred to the stigma of emasculated flowers. Similarly self-pollination was done at the same time and in the same fashion for all the parents. Crosses were made between female and male parents under field condition to calculate percentage of pod set. The pollen fertility was assessed in three parents using 1% I$_2$-KI solution. The stained pollen grain was considered as fertile whereas unstained as sterile.

Table 2. Pollen tube growth in *V. radiata*, *V. mungo*, *V. umbellata* and their crosses

<table>
<thead>
<tr>
<th>Genotypes/Crosses</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>18</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. radiata</em> selfed</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td><em>V. mungo</em> selfed</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td><em>V. umbellata</em> selfed</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td><em>V. radiata</em>×<em>V. umbellata</em></td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>V. mungo</em>×<em>V. umbellata</em></td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Where, --, pollen grains not germinated on stigmatic region; +, pollen grains germinated on stigmatic region; ±, inhibition of pollen tube growth either due to stigmatic exudation or bend back of pollen tube growth on stigmatic region; ++ pollen tube growth in the upper stylar region; +, inhibition of pollen tube growth due to degeneration or lateral tube growth in the upper stylar region; ++++, pollen tube growth in the middle stylar region; +++±, minimum number of pollen tube reached in the middle stylar region; ++++, pollen tube growth in the bottom stylar region; ++++, pollen tube growth in the upper stylar region; +++, pollen tube growth in the middle stylar region; ++, pollen tube growth in the bottom stylar region; ++, pollen tube growth in the upper stylar region; +, pollen tube reached the ovary; +++, pollen tube reached the ovary and fertilized.

Fig. 1. Time taken for the growth of pollen tube in parents and crosses of *Vigna* spp.
The pollinated flowers were collected after 2, 4, 6, 8, 10, 12, 18 and 24 h after pollination (HAP) and were fixed in acetic acid, alcohol (1:3 v/v) fixative. The pistils were separated from the flowers and stored at 4°C for 12 h. For every collection time, 10 crossed and self-pollinated flowers each from five tagged plants of *V. radiata* and *V. mungo* were used for microscopic observations. The methodology described by Parani (1998) for Sesame was modified slightly for *Vigna* with respect to softening period with sodium hydroxide (NaOH) and tri basic potassium phosphate (K$_3$PO$_4$).
After fixation, the pistils were washed with distilled water and treated with 6 N NaOH solutions for at least 9 h to clear and soften the tissue. The softened pistils were put in distilled water for 1–2 h, giving 4–5 washings and then stained with 0.1% water-soluble aniline blue prepared in 0.1 M K$_3$PO$_4$ for 24 h. The stained pistils were mounted in a drop of 50% glycerol on a glass slide and were covered with cover slip, which was pressed gently into a thin layer. The slides were kept in dark until observed under a dark field microscope (Olympus BX 60) with fluorescent attachment using 390–420 nm barrier filter coupled with a 450 nm excitation filter. The pollen tube growth was observed and images were captured with an Olympus digital camera model E500.

Results

The crosses were made between *V. radiata* cv CO 5 and *V. mungo* cv VBN1 (as female) and *V. umbellata* (as male) under field condition to produce hybrids. The crosses between *V. radiata* and *V. umbellata* showed 12.89% pod set whereas *V. mungo* and *V. umbellata* recorded 5.56%. *V. radiata*, *V. mungo* and *V. umbellata* parental species noticed 90.25%, 89.15% and 88.55% pollen fertility respectively, this is normal to effect fertilization.

The *in vivo* pollen tube growth study was observed in selfed-parents as well as crosses under fluorescence microscope. The pollen grain germination upon self-pollination in parental species was normal at the stigmatic region (Fig. 1, Table 2). In *V. radiata*, pollen tubes passed through the stigmatic surface within 2 HAP, and reached the bottom stylar region within 8 HAP, then reached the ovary and get fertilized within 10 HAP (Fig. 1, Plate 1a–c), and similar observations were also observed in *V. umbellata* (figure not shown). In *V. mungo*, pollen tubes passed through the stigmatic surface and reached upper stylar region within 2 HAP, the bottom stylar region reached within 6 HAP, then reached ovary and get fertilized within 8 HAP (Fig. 1, Plate 1d–f).

Germination of *V. umbellata* pollen grains upon pollination with *V. radiata* as well as *V. mungo* was normal. In cross between *V. radiata* and *V. umbellata*, the rate of pollen tube growth was reduced at all levels in *V. radiata* pistils (Fig. 1, Table 2). Pollen tube passed through the stigmatic surface only at 4 HAP, and even at 10 HAP growing pollen tubes were still observed in the mid stylar region (Table 2). Inhibition of pollen tube penetration and accumulation of stigmatic exudation in stigmatic region (Plate 1g, Table 2) were observed. The bursting and degeneration of pollen tubes were also commonly observed in upper stylar region (Plate 1h, Table 2). The minimum number of pollen tube reached the ovary at 12 HAP and fertilization taken place within 18 HAP (Plate 1i, Table 2).

The rate of pollen tube growth was drastically reduced in all stages of *V. mungo*×*V. umbellata* cross and most frequently in stigmatic region (Fig. 1, Table 2). The pollen tube passed through the stigmatic region only at 8 HAP, and the pollen tube penetrating even at the upper stylar region 10 HAP (Fig. 1). The alien pollen tubes growing with reverse direction towards the stigma were observed (Plate 1j, Table 2). Pollen tubes with swollen and collapsed pollen tubes were frequently noticed (Plate 1k, Table 2). Within 18 HAP, the reduced number of pollen tubes passed to the ovular region and effected fertilization to minimum number of ovules in 24 HAP (Plate 1l, Table 2).

Discussion

Fertilization between diverse species within the same genus will be successful only if there is some means of compatibility between both the parents. The successful completion of a series of sequential events following pollination requires a perfect blend of co-ordination between genes and gene complexes of pollen and the ovule parents (Hogenboom 1973). The present investigation was mainly focused on the rate of pollen tube growth and structural abnormalities of alien pollen on the cultivated pistils to understand how the reproductive isolation barriers operate on pollen tube pene-
tration in different stages of pistils. The divergent relative species namely *V. umbellata* confers more durable resistance to insect pests and diseases (Watanasit and Pichitporn 1996). In the present study, low pod set was observed in *V. radiata* × *V. umbellata* (12.89%) and *V. mungo* × *V. umbellata* (5.56%) crosses. However the pollen fertility was found to be normal. Low pod set observed in the above crosses could be due to the operation of pre-fertilization barriers. Similar results were noticed in the inter-specific crosses of *Vigna* species (Umamaheshwari 2002).

In *V. radiata* × *V. umbellata* cross, the pre-fertilization barriers at all levels of tissue were significant, as indicated by moderate pollen tube penetration and growth. The slow rate of pollen tube growth was noticed in inter-specific crosses of rice (Sarker *et al.* 1983) and maize (Manickam 1996). Although the pollen tube growth was slowed down by the structural aberrations like stigmatic exudation and degeneration of pollen tubes (Plate 1g and h), eventually pollen tube penetrated into the bottom stylar tissues and reached the ovary. Similar types of structural malformations were reported in Cotton (Gunasekaran 1997). Umamaheshwari (2002) reported low percentage of pod set in the same cross using different accessions of parents. The techniques such as pollen mixture, application of growth hormones and protoplast fusion may rectify the defects before fertilization and lead to development of new inter-specific hybrids from related species.

The pre-fertilization barriers were predominant in *V. mungo* × *V. umbellata* cross and operated in all the stages as delayed or inhibited penetration of pollen tube growth and disorientation of pollen tubes in stylar tissues. The pre-fertilization barriers were not restricted to any one particular stage of development, but had operated very gradually and mildly in varying degree. The common pre-fertilization barriers found to occur in many wide crosses are pollen-pistil incompatibility. These barriers are influenced by delayed pollen grain germination and pollen tube growth of one species on the stigmas of another species (Monika *et al.* 2005, Hodnett *et al.* 2005).

Events starting at pollination and terminating at fertilization in wide crosses involve complex and harmonious interactions between the microgametophyte and the sporophyte of the pistil parent. It is understood that proper recognition of protein substances, pollen tube penetration on the stigma and co-ordination between the pollen and pistil proteins are necessary to effect normal pollen tube growth. When pollen grains succeed in crossing the stigmatic surface, growing pollen tubes are guided to the micropyle by signals originating in the style and embryo sac (Lord and Russell 2002). During discordant situations, normal metabolism of the pollen tube was obstructed and pollen tube collapsed, reducing further growth to the micropyle. Hodnett *et al.* (2005) indicated that adverse pistil-pollen interactions that include the pollen tube growth inhibition in wide crosses, which might be viewed as consequence of inharmonious genetic interactions due to genetic divergence of the species involved.

In the present study, apart from the slow rate of pollen tube growth several other types of aberrations were observed in *V. mungo* × *V. umbellata* cross. Alien pollen tubes (*V. umbellata*) typically had shown a reverse direction of growth path towards the apex of stigma (Plate 1j). Such irregularities in pollen tube development were furnished in pistils of cultivated sorghum with the pollen tube of *Sorghum intrans* and *S. interjectum* wide crosses (Hodnett *et al.* 2005) and also in *Gossypium* spp. (Gunasekaran 1997). Another common aberration of alien pollen tube growth in cultivated *Vigna* pistils observed in this study was the lateral expanded or swollen pollen tubes in stylar tissue (Plate 1k). This phenomenon has been found in other species as well. The cross between wheat (*Triticum aestivum*) and rye (*Secale cereale*), swollen rye pollen tubes has been noticed in wheat pistils (Jalani and Moss 1980). Wild species of Sesame (*Sesamum laciniatum*) swollen tube and a twisted growth pattern was found in cultivated Sesame (*Sesamum indicum*) style (Ganesh Ram *et al.* 2006). Although some degree of abnormalities observed in the present study namely slow rate of pollen growth and structural aberrations, the least number of pollen tubes were reached the embryo sac and fertilized one or two ovules. It was evidenced by the presence of few seeds in the crossed pod. Low seed set per pod was observed in crosses of different *Vigna* species (Umamaheshwari 2002).
It was suggested to use techniques such as bud pollination, application of growth hormones, in vitro fertilization and protoplast fusion (Chen et al. 1989) to promote pollen germination, and pollen tube penetration in stylar and ovular tissues to effect fertilization in the wide crosses with pronounced pre-fertilization barriers. Hence, the use of one or a combination of these techniques could be utilized for the production of inter-specific hybrids and introgression of MYMV and bruchids resistance genes from the V. umbellata to the cultivated V. radiata and V. mungo.

References