dried as they contained more moisture in the form of mucilage. The pressed and dried specimens were autoradiographed by placing on X-ray films in the dark and covered with smooth paper and pressed. The X-ray films were exposed for 10 days in the press. The plant parts were removed and the film was developed using a commercial X-ray film developer solution.

The autoradiograms revealed varied results. The isolates EB-35 (*B. subtilis*) and EB-65 (*P. aeruginosa*) gave positive result when applied on leaves and pods, whereas the results were negative for the other two isolates (EB-31, *P. putida* and EB-40; *Pseudomonas* sp.). From Figure 2 *a* it is clear that the radioactive bacteria reached the conductive tissues of shoots and leaves which are situated above the treated leaf. The darker image of the lower leaf shows tagged bacteria at the site of application. Figure 2 *b* reveals the presence of labelled bacteria in the placenta of the pod. The results indicate that the endophytic bacteria are capable of entering the host tissue through intact surface of leaves and pods and move through the conducting tissues. Entry of bacteria through stomata is a well-known fact, and this study suggests the capacity of these novel biocontrol agents to establish at the site of infection of the pathogen thereby offering effective protection when they are applied on the plant surface. However, in the present study, labelled bacteria could not be detected in the shoot portion of the plant when applied on the roots and also on the collar region. The possible reason for this is that the bacteria could not reach up to the shoot within the period of exposure (48 h) given. The result was negative for two isolates which may be due to their inability to tolerate the radioactivity to which they are exposed, which might have caused attenuation of systemic movement. Previously, radiolabelling has been used successfully to detect the entry and movement of endophytes[6]. This technique has been used for studying the mode of entry and spread of *Ralstonia solanacearum* in tomato seedlings[8]. Autofluorescent protein (AFP) methods are being utilized to detect and enumerate endophytic microorganisms and to study the ports of entry to plants[9]. However, radiolabelling is comparatively less laborious as there is no need of transforming the bacteria as in the case of the AFP method. This is quick and colonization of EB within the tissues can be viewed on the radiogram.

1. Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E. R. B., Taghavi, S., Mezeay, M. and van der Lelie, D., *Crit. Rev. Plant Sci*., 2002, **21**, 583–606.
2. Kloepper, J. W., Ryu, C. M. and Zhang, S., *Phytopathology*, 2004, **94**, 1259–1266.
3. Aravind, R., Kumar, A., Eapen, S. J. and Ramana, K. V., *Lett. Appl. Microbiol*., 2009, **48**, 58–64.
4. Melnick, R. L., Ph D dissertation. The Pennsylvania State University, USA, 2010.
5. Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J. and Dowling, D. N., *FEMS Microbiol. Lett*., 2008, **278**, 24–33.
6. Pleban, S., Ingel, F. and Chet, I., *Eur. J. Plant Pathol*., 1995, **101**, 665–672.
7. Mc Inroy, J. A. and Kloepper, J. W., *Plant Soil*, 1995, **173**, 337–342.
8. Markose, L. M., Ph D thesis, Kerala Agricultural University, Thrissur, 1996, p. 140.
9. Tombolini, R., Unge, A., Davey, M. E., de Bruijn, F. J. and Janson, J. K., *FEMS Microbiol. Ecol*., 1997, **22**, 17–28.

Received 30 March 2012; accepted 8 August 2012

P. SAINAMOLE KURIAN*
KOSHY ABRAHAM
P. SURESH KUMAR

College of Horticulture,
KAU (PO), Vellanikkara,
Thrissur 680 656, India
**For correspondence.*
e-mail: sainasajayan@yahoo.com

Pollination without emasculation: an efficient method of hybridization in soybean (*Glycine max* (L.) Merrill)

Hybridization is the primary step towards generating segregating populations. However, successful hybridization involves both science and skill. Success of the crosses varies from crop to crop. It also varies with the season, crop health, weather conditions and the variety of the crop used. Broadly speaking, compared to cereals, success of crossing is less in grain legumes, including soybean, green gram, black gram and chickpea. In these crops, natural flower dropping adds to the menace. In soybean, the success of artificial crosses varies from 2–3% to 11–15% in field conditions[1]. The reason for such variation lies mainly with the approach followed for crossing. Normally, the soybean breeders perform hybridization through manual emasculation (in the evening) followed by pollination (the next day morning). Some breeders prefer simultaneous emasculation and pollination. Here, we report an approach that is devoid of emasculation. It ensures less damage to the flower buds and hence more success.

For making successful crosses, it is essential to know the flower well. Soybean belongs to the family Fabaceae and sub-family Papilionoideae. It has a complete flower, i.e. all the four parts, viz. calyx, corolla, androecium and gynoecium are present in a single flower (Figure 1). The five petals – standard (one), wings (two) and keels (two) enclose the pistil and the 10 stamens. Nine stamens develop in a tube around the pistil, the tenth stamen remains free. Pollen from the anthers is shed directly on the stigma. Often, pollen is shed shortly before or immediately after the flower opens (anthesis). It ensures a high degree of self-pollination and less than 1% natural cross-pollination.

Soybean flower is very small and delicate; it drops even with minor injuries to the pistil. Therefore, during artificial crossing utmost care should be taken not to injure the pistil. Usually during crossing, the anthers are carefully removed from the bud (the process is called emasculation) selected for crossing (recipient parent); it is then pollinated with anthers collected from the flowers of a donor

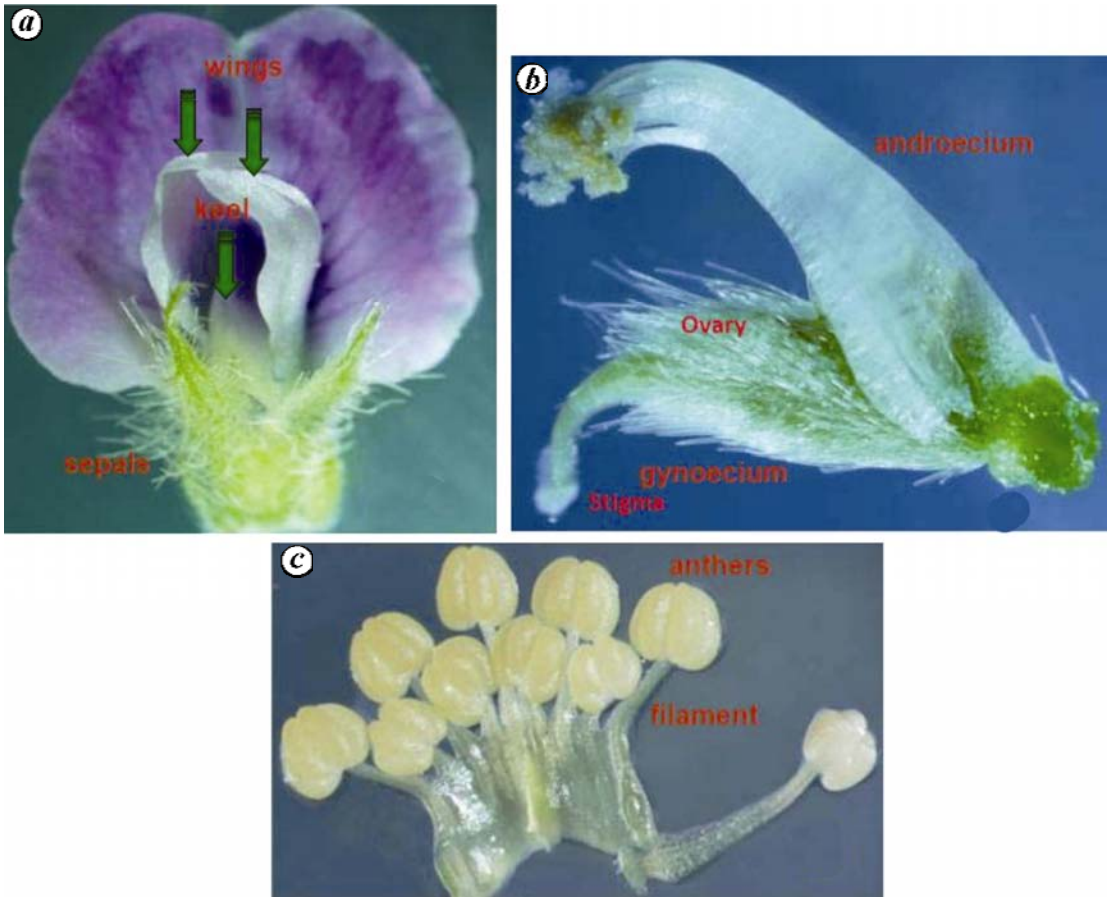

Figure 1. ***a***, Five petals of soybean flower. ***b***, Androecium and gynoecium. ***c***, Filaments and anthers.

parent. Emasculation eliminates chances of self-pollination; however, it is injurious to the flower bud. Here we propose a method of crossing called pollination without emasculation. The basis of this method lies in the biology of the flower. In fact, soybean flower is protogynous in nature[2], i.e. the stigma becomes receptive to pollen one day before the anthers begin shedding pollen. Therefore, if a flower bud is identified before its pollen grains are shed, the emasculation step can be avoided. So, pollination can be done without emasculation. The pollen germinates within 24 h of pollination. This principle was effectively used in soybean hybridization and the rate of success was raised from 2–3% to around 39% in the field conditions in Delhi, India. The various steps and special considerations are explained below.

Soybean flowers are very small and need extra care. Therefore, for effective crossing, it is necessary to use forceps with fine tips. Moreover, headmounted (or hands-free) magnifying glass has proven to be useful. It makes every part of the flower clearly visible ensuring little or no damage during pollination.

It is critical to identify a flower bud that would bloom the next day morning. It is generally identified by the size and colour of the buds. The buds ready to open are larger in size and relatively lighter in colour than the immature buds. The petals are not exposed out of the bud. Usually, 1–3 such buds appear in each cluster. Once identified, rest of the buds, i.e. those already opened and immature buds should be removed from the cluster.

The selected buds should be held softly between the first finger and the thumb. With a fine forceps, the sepals are to be removed carefully. The petals should be opened (or removed) to expose the ring of stamens that surrounds the pistil. Locating the pistil makes pollination easy and effective.

Choosing anthers at the proper stage of development is crucial for obtaining high seed set. It is therefore important to use mature pollen to pollinate the flower buds. Pollen should be collected from fully opened fresh flowers only. The mature pollen come out of the anthers as yellow dust.

Once the flower bud is prepared and the pollen grains are collected, pollination should be done immediately by distributing the pollen on the stigma. Care should be taken to ensure that the pollen falls on the stigma. To prevent drying of the stigma, the buds may be covered with a thin layer of moist cotton.

Time for effective hybridization may vary with the growing season and the weather conditions. During kharif, it can be performed between 8.30 and 10.30 a.m. However, during cold season, flower opening and pollen shedding is delayed by about half to one hour. In controlled condition (like phytotron facility), crossing can be performed from 8.30 to 11.30 a.m.

After crossing, proper tagging should be done. The plants should be given proper water and fertilizer to avoid stress. The uncrossed flowers and young buds should be removed from the plants.

Success of crossing can be judged 4 days after pollination. Upon successful

Table 1. Result of crossing without emasculation in different generations of soybean over the years

	Kharif 2010 (field)		Rabi 2010 (NPF)*		Kharif 2011 (field)	
	Cross 1**	Cross 2	BC_1 of cross 1	BC_1 of cross 2	BC_2 of cross 1	BC_2 of cross 2
Flower buds crossed (no.)	130	135	182	155	175	85
Buds developed into pods (no.)	50 (38.46%)[$]	45 (33.33%)	45 (24.72%)	35 (22.58%)	62 (35.42%)	19 (22.35%)
Seeds harvested (no.)	85 (1.7)[#]	72 (1.6)	72 (1.6)	59 (1.7)	105 (1.7)	32 (1.7)
Pure hybrid plants (no.)	78 (74.12%)[§]	53 (73.61%)	55 (76.89%)	45 (74.58%)	80 (76.19%)	23 (71.87%)

*NPF: National Phytotron Facility. **Cross 1: DS9712 × PI542044; Cross 2: DS9814 × PI542044. [$]Figures in parenthesis indicate the success of crossing (%). [#]Figures in parenthesis indicate the average no. of seeds/pod. [§]Figures in parenthesis indicate production of true hybrid plant (%).

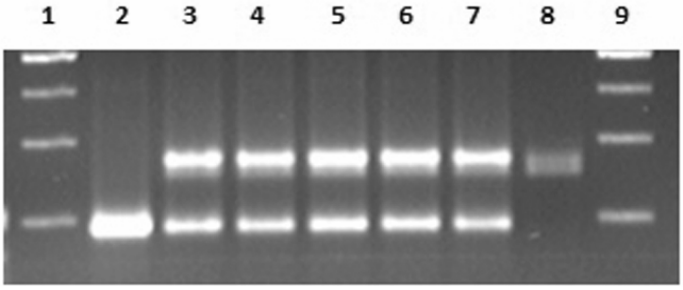

Figure 2. Hybridity testing through SSR markers. Lanes 1, 9: Marker; lane 2: Parent 1; lane 8: Parent 2; lanes 3–7: F_1 hybrid plants.

crossing, the bud remains green and starts growing; otherwise it dries up and drops off the plant. The plants need to be monitored every week to remove newly grown buds.

The crossing approach described above was utilized in two intra-specific (*Glycine max* × *G. max*) crosses of soybean, viz. DS9712 × PI542044 and DS9814 × PI542044. In both the crosses pollination was done without emasculation according to the steps mentioned above. Percentage of bud survival and development into full-grown pods ranged from 33% to 38% in F_1 generation and 22% to 35% in backcross generations (Table 1). It was significantly higher than the success (11.53–14.72%) obtained through traditional approaches[1] (emasculation followed by pollination). Success of crossing during rabi season was less compared to kharif because of prolonged cooler temperature with shorter day length that leads to the development of more cleistogamous flower[3]. Such flower buds usually do not exhibit proper developmental stage, making it difficult to identify the right buds for crossing.

True success of this approach came through the production of higher number of true hybrid plants from the crosses in every generation. The hybridity of the F_1 plants was established through polymorphic SSR markers (Figure 2). The number of true F_1 hybrid plants was 78% and 72% in the crosses 1 and 2 respectively. This was also significantly higher than the reported approach with 64% success through emasculation followed by pollination[4]. In the BC_1 and BC_2 generations, the success was more pronounced (> 70%; Table 1). This can be attributed to the extra care taken during crossing as well as the limited damage caused to the buds. While selecting the buds, it should be carefully verified that they are not already self-pollinated. This would avoid the number of self-pollinations.

It was further observed that the rate of success varied between crosses 1 and 2. Although the male parent was common (PI542044) in both the crosses, more success was observed in all the crosses involving DS9712 as the female parent. It showed that success of crossings also depends upon the genotype involved (Table 1). The result thus showed that pollination without emasculation does not increase incidence of self-pollination, rather it increases the success of crossing through limited injury to the flower buds.

Thus we have shown that pollination without emasculation is an effective approach for crossing in soybean. It ensures more bud survival and more number of true hybrid plants. Thus, it is a robust method of crossing in self-pollinated crops like soybean.

1. Agrawal, A. P., Ravikumar, R. L., Salimath, P. M. and Patil, S. A., *Indian J. Genet.*, 2001, **61**, 76–77.
2. Walker, A. K., Cianzio, S. R., Bravo, J. A., and Fehr, W. R., *Crop Sci.*, 1979, **19**, 285–286.
3. Grinev, A. K. and Kochegura, A. V., *Slektsiya I Semenovodstvo* (*Moscow*), 1988, **3**, 10–12.
4. Vollmann, J., Gruber, H., Gretzmacher, R. and Ruckenbauer, P., http://www.boku.ac.at/diebodenkultur/volltexte/band-43/heft-1/vollmann.pdf

ACKNOWLEDGEMENT. We thank Dr Ram J. Singh, Department of Crop Sciences, National Soybean Research Laboratory, University of Illinois, Urbana, USA for guidance and for also providing the photographs.

Received 14 March 2012; accepted 31 July 2012

AKSHAY TALUKDAR*
M. SHIVAKUMAR

Division of Genetics,
Indian Agricultural Research Institute,
New Delhi 110 012, India
**For correspondence.*
e-mail: atalukdar@iari.res.in