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Endophytic bacteria – do they colonize within the plant tissues if applied externally?

The intimate association of endophytic bacteria (EB) with plants makes them potential candidates for biological control. Many bacterial species were reported to form endophytic associations with plants[1]. The beneficial effects that these bacteria confer on plants have made the study of plant–endophyte associations an important research topic for studying biological control of plant diseases[2]. Conventional biocontrol agents are not effective in the management of aerial plant pathogens mainly because of the lack of medium for survival and multiplication on the plant surface. Hence they may get washed away before they can act on the plant. This difficulty is solved to a considerable extent by the use of endophytic microbes which enter through natural openings and reside within the plants. They use plant sap as a medium for growth and systemic movement. Many workers have reported the bio-control efficiency of EB in different patho-systems[3,4]. Endophytes have been isolated from practically all studied plants following standard protocols. But their re-entry has not been proved in most cases.

Endophytes originate from the rhizosphere[5] and some may be transmitted through seeds and natural openings[6]. But if they are applied externally as other biocontrol agents, do they re-enter the plants through natural openings? The best way to find out this is by the radiolabelling technique which gives visual proof for the entry and colonization of labelled endophytes inside the plants. An experiment was conducted using four-month-old cacao seedlings and half matured cacao pods collected from 10-year-old cacao trees. Four bacterial endophytes isolated from different parts of cacao trees by surface sterilization followed by trituration as described by Mc Inroy and Kloepper[7] were used for the experiment.

For isolation of endophytes from feeder roots, tender shoots or leaves, the 1 g samples was exposed to the sterilant followed by washing with three changes of sterile water. The root/shoot/leaf bits were then put in a sterilized mortar containing 10 ml sterile potassium phosphate buffer (PB; 0.1 M, pH 7). The bits were finally washed in the buffer. From the final buffer wash, 1 ml was pipetted out and poured into sterile petri plate. To this molten and cooled medium was added and it served as the sterility check. If microbial growth was observed in the sterility check within four days, the isolates obtained from that particular sample were discarded.

For isolation from cacao pods, half mature pods collected were washed and disinfested with 70% ethanol. They were then cut open aseptically using sterile scalpel. A small piece (1 cm^3 approximately) of placenta of the pod was taken and placed in a pre-weighed, sterile petri dish. The petri dish was weighed along with the piece of tissue. The weight of the tissue was thus calculated and used to find the number of colonies per gram.

The surface-sterilized bits of root/shoot/leaf or the placenta of pod were triturated using sterile mortar and pestle with 9 ml of sterile buffer[7]. The triturate was serially diluted in sterile PB up to 10^{-7}. Then 1 ml of the diluted triturate was pipetted into sterile petri plate and molten and cooled King's B agar medium was added. The plates were

incubated at 28 ± 2°C for 48 h. Representative colonies of EB based on colony morphology were picked up from the dilution plates and transferred to slants to establish pure cultures following standard protocols. Altogether, isolates of 116 bacteria and 153 fluorescent pseudomonads were thus subcultured. These were subjected to various *in vitro* and *in vivo* screening and the most efficient endophytic antagonists were selected which were used in the present experiment. These selected isolates were identified by biochemical tests as well as by 16S rDNA sequence comparison.

The experiment was carried out at the Radiotracer Laboratory (RTL), College of Horticulture, Vellanikkara, Thrissur, Kerala. The radioisotope ^{32}P was procured from the Board of Radiation and Isotope Technology, Mumbai. Pure cultures of the bacterial endophytes were prepared on solid medium. The medium used was King's B agar for three fluorescent pseudomonads, viz. EB*-31 (*Pseudomonas putida*), EB-40 (*Pseudomonas* sp.) and EB-65 (*Pseudomonas aeruginosa*) and nutrient agar for EB-35 (*Bacillus subtilis*). Log-phase cultures of the endophytic bacteria were used for radiolabelling. For preparing labelled inoculum, 41 µCi ^{32}P ml^{-1} was added to sterilized liquid medium (King's B). One loop full of the log phase culture was aseptically transferred to 50 ml of the labelled liquid medium and incubated for 48 h at 28°C. The growth medium with radioactivity was removed and the cells were pelletted by centrifugation in order to assure that the radioactivity that may be detected in the inoculated plant is from the bacteria which had entered into it. The labelled broth culture was centrifuged at 5,000 rpm for 15 min. The supernatant was removed and the bacteria pelletted at the bottom of the centrifuge tube was resuspended in sterile water and centrifuged again. This was repeated thrice and the supernatant was tested for the presence of radioactivity in a liquid scintillation counter. Centrifugation and re-suspension in fresh sterile water was repeated until the supernatant was free from radioactivity. Finally the labelled bacterial pellet was re-suspended in 25 ml sterile water and used for plant inoculation. This inoculum used for the experiment had 10^7 cfu ml^{-1} labelled bacteria.

Inoculation with labelled bacteria was done on cacao seedlings and pods. On two-month-old seedlings, inoculation was done at three different sites, viz. roots, leaves and basal portion of the stem. In all the three methods, inoculation was done without giving injury. For inoculation of roots, ^{32}P-labelled bacterial suspension (1.5 ml) was taken in small plastic tubes. Actively growing feeder root was carefully excavated, without breaking. The tip of the root was washed to remove adhering soil and was placed carefully in the bacterial suspension in the tube (Figure 1 *b*). For the leaves the bacterial suspension (500 µl) was applied on the upper surface of the third leaf of a new flush as small droplets using a micro-pipette and allowed to air-dry. On the stem, cotton wool wet with 500 µl bacterial suspension was placed at the collar region.

Half mature cacao pods collected from a 10-year-old cacao tree were used for inoculation. The ^{32}P-labelled bacterial suspension (500 µl) was carefully applied into the depression around the pedicel using a micropipette and allowed to air-dry (Figure 1 *a*). Three replications were maintained for each bacterium. The inoculated plants and pods were kept in the greenhouse attached to RTL for two days, in order to allow the labelled bacteria to enter and colonize the tissues.

Two days after inoculation, the aerial plant parts of the cacao seedlings were detached by cutting at the base of the stem in the case of root and leaf-inoculated plants. In the case of plants inoculated on the stem, it was cut at 3 cm above the point of inoculation. For pods, the inoculated part was cut and removed from the pods using a sharp knife. Then three thin slices were taken from the pod, one from the centre and the other two from the two sides. The plant parts were then arranged on absorbent paper in their original position, labelled and secured with adhesive tape. The specimens sandwiched between absorbent sheets were then pressed in a herbarium press and allowed to dry at room temperature. This will remove the moisture from the specimens which will attract microbes that may damage the photosensitive coating on the X-ray film. Specimens taken from the pods were dried in a hot-air oven for two days at 50°C and then air-

Figure 1. Application of ^{32}P-labelled endophytic bacteria on cacao pods (***a***) and roots (***b***).

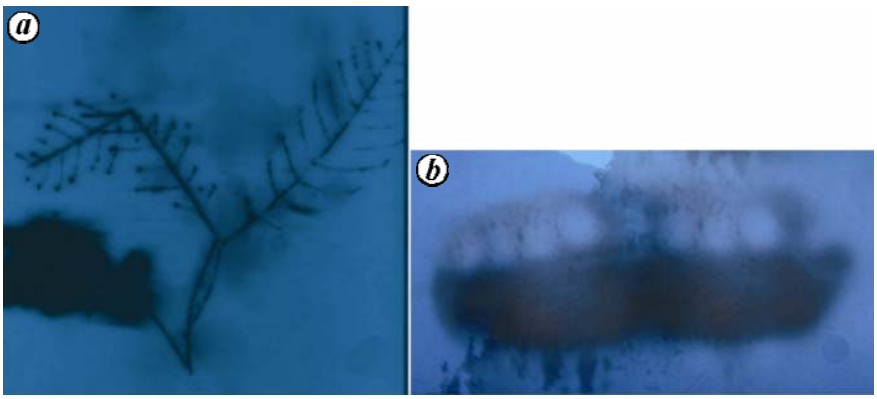

Figure 2. Autoradiogram showing ^{32}P-labelled EB-35 (*Bacillus subtilis*) in the conductive tissues of cacao seedling (***a***) and EB-65 (*Pseudomonas aeruginosa*) inside cacao pod (***b***).

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.

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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