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Methods for Introduction of the Atmospheric Nitrogen Fixing Ability to Plants

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Abstract

An artifical symbiosis was established between diazotropic *Azomonas insignis* and strawberry (*Fragaria x ananassa*). The partnership was created by in vitro techniques through callus induction and organogenesis. The basis of this partnerships is the bacterial dependence on the plant,s metabolic activity,using maltose in the medium as a carbon and energy source which can be utilized by the plant cells only.

The presence of bacteria in the intercellular spaces of the callus tissues and regenerated plants was proven by microscopic techniques. Nitrogenase activity could also be detected in the plant tissues.

For successful and high frequency introduction of bacteria to the plant tissues, biolistic gun method was used. On the basis of the DNA transfer method, *Azotobacter vinelandii* bacteria were delivered directly into strawberry tissues by the particle bombardment. This was the first use of living bacteria as microprojectils for bombardment of plant tissues. The treatment was successful, the presence of bacteria in the developing callus tissue and regenerated plants were detected by light and electron microscopy.

Introduction

Nitrogen shortage is the main limiting nutrient in agricultural practice because only some procaryotes can fix atmospheric nitrogen by means of their nitrogenases. These diazotrophs are sometimes abundant in terrestrial or aquatic ecosystems and their contribution to nitrogen supply is significant even in agricultural areas.

Some procaryotes, so called free living types (aerobic *Azotobacters*, anaerobic Clostridium, blue-green algae as *Nostoc*) are able to fix N_2 without cooperation with other organisms while others (*Anabaena, Rhizobium, Azospirillum*) fix atmospheric nitrogen only in association with plants. The organization of the latters ranges from loose associations to more integrated partnership. On the basis of natural symbioses, several attempts were made to establish artificial symbiosis between suitable diazotrophic procaryotes and plants [15]. The factors affecting the succesul establishment of such N_2-fixing systems are complex, but depend on at least five major factors: the specificity of associations, a fairly steady ratio between the biomass of the eukaryote in the symbiosis, and morphological, physiological and biochemical modifications of the partners.

In the forced assotiation of free living adenine-requiring mutant of *Azotobacter vinelandii* with tissue culture of *Daucus carota*, the nitrogen fixing bacteria were living among the callus cells [16]. Partial coordination of metabolic functions was indicated by enhanced ethylene production and prolonged stability of the mixed calli but plant regeneration did not occur.

Sugarcane callus cultures were inoculated with nitrogen fixing *Azospirillum* brasilense and nitrogenase activity was detected in the co-cultures up to 18-20 months. However the plantlets regenerated from mixed calli did

not contain bacteria [1, 26].From a mixed culture of carrot cells and Azotobacters, somatic embryogenesis could be induced and the regenerated plants contained bacteria in the intercellular spaces [17, 25]. Acetylene reduction was also detected in potted plants.

There were attempts to create artificial nitrogen fixing intracellular symbioses too. Giles and Whitehead [7, 8] introduced *Azotobacter vinelandii* into *Rhizopogon* fungal protoplasts and nitrogenase activity was observed up to seven months. Tobacco and maize protoplasts took up diazotrophic cyanobacteria *Gloecapsa* [3, 4] but they harbored generally on large algal cell and the rate of uptake was less than 1%. Endocytobiosis was created by PEG induced fusion of cell wall mutans of the unicellular green alga, *Chlamydomonas reinhardtii.* The bacterial cells were taken up by algal cells during the fusion [10]. This artifical system was maintained on nitrogen and carbonhydrate free medium for a long time and the bacterial cells were always found inside some of the algal cells. The other form of *Clamydomonas-Azotobacter* association was the simple exocytobiotic cocultivation of these partners where the bacteria were present among the cells of the algal colonies [16]. This exocytobiotic association proved to be more stable and effective system in comparison with poorly viable endocytobiotic system.

On the basis of these results we tried to introduce bacteria into multicellular plants. *In vitro* methods were used for bacterium incorporation through callus induction from inoculated leaves of strawberry [21] and from co-cultured carrot cell suspension [25]. In both cases bacterium incorporation into the intercellular spaces of callus and regenerated plants is a random process as well as their transmission from generation to generation in the course of vegetative propagation.

A new method allows for a higher incidence of bacterium introduction into the treated plant inocula. This method is the particle borbardment. The main field of use of the biolistic gun is the genetic transformation of different organizms where plasmid DNA is adhered to tungsten or gold particles, and shot into target inoculum. Only Rasmussen et al [23] reported on the use of phenol pretreated dead bacterial cells as microprojectiles so as to deliver DNA into target cells. In contrast to this, we aim to introduce living bacteria into the plant tissues by particle bombardment to create a functioning artificial symbiosis between Azotobacters and strawberry [22].

This work is summarizes the technologies-used by us- to establish artificial plant-microbe associations for atmopheric nitrogen fixation.

Materials and Methods

The bacterial partners

Aerobic, free living, Gram negative N_2 fixing bacteria were used. The CCM 289 strain of *Azotobacter vinelandii* and the CRS-HK 5 strain of *Azomonas insignis* were maintained in nitrogen free liquid and solid culture (20) at 30℃ in the dark.

The host plant

Shoot cultures of strawberry (*Fragaria x ananassa* "Fertõdi F5") were initiated from virus-free shoot tips and maintained on MS basal medium (19) and supplemented with 0.5 mg l^{-1} BA, 0.1 mg l^{-1} GA3, 0.5 mg l^{-1}IBA and 3% sucrose. The pH was adjusted to 5.7 before adding 0, 6% (w/v) bacteriological agar (Merck) and autoclaving for 20 min at 121℃ and 108 kPa. Cultures were incubated at 25℃ and a PPF of 40 mol $m^{-2}s^{-1}$ (Tungstram F 29, warm white tubes) under a 16 h photoperiod. Individual plantlets were transferred to fresh medium at 6 week intervals.

Establishment of plant - Azomonas coculture via callus culture and plant regeneration

Callus cultures were initiated from micropropagated shoot cultures. The youngest expanding leaves were exised from 1 month old shoots and the petioles removed. Leaves were placed on callus inducing medium (MS basal medium supplemented with 1 mgl^{-1} BA, 0.1 mgl^{-1} IBA, 0.05 mgl^{-1} NAA, 0.1 mgl^{-1} 2.4-D, 3% maltose and 0. 6 agar pH was adjusted to 7.0) with both abaxial and adaxial sides touching the medium. The regeneration media was the same as the callus initiation media.

In other experiments cell suspensions were obtained from calli. This cell suspension was mixed and incubated with *Azomonas* cells for overnight. On solid medium as above, callus tissues were obtaind again.

Rooting and soil culture

Shoots, regenerated from callus, were excised when

they were 10 mm in length. The rooting medium was MS basal medium without any growth regulator. Rooting conditions were the same as were used at the micropropagation stage. After 4 week, the plantlets were transferred to pots containing nutrient-poor soil mixture (perlite:peat:compost 2:1:1 by volume). Five rooted strawberries were placed into the 500 ml PE VegBox-es. Later, as the plants developed, the samples was reduced to two per pot.

Particle transfer of bacterial cells

Tungsten particles (Biorad M25) of 1.7 μm average size were suspended in ethanol at 60 mg/ml concentration and stored at -20℃. For bombardments, 25 μl aliquots were taken, washed out thoroughly by several volumes of sterile water and mixed with bacteria in 165 μl sterile water at about 10^9 cells/ml. Following vigorous vortexing, 5 μ l spermidin (0.1 M) was added to the suspension, which was then put on ice for 10 min. to let the particles sediment. After removing 130 μl of supernatant, the mixture was briefly vortexed and 5 μl aliquots were pipetted onto the plastic macroprojectiles which were accelerated by a nitrogen powered Genebooster biolistic gun at high pressure (33-35 bars). The shot distance was about 12 cm.

Microscopy

For light and transmission electron microscopy, tissue pieces were fixed in 2% glutaraldehyde for 2 h and postfixed in 1% OsO_4 for 2 h. Buffer solution was 70 mM K-Na phosphate (pH 7.2). Samples were embedded in Durcupan ACM epoxy resin (Fluka Chemie AG). Semithin sections were stained with toluidine blue. Ultrathin sections were stained with uranyl and lead salts and observed with a Hitachi 7100 electron microscope.

For scanning electron microscopy, small parts of leaves were fixed in 2% glutaraldehyde for 3 h and postfixed in 1% OsO_4 for 3 h. Buffer solution was 70 mM K-Na phosphate (pH 7.2). After dehydration with inreasing concentrations of ethanol, samples were placed in amyl acetate. The intermediate fluid was removed from the samples by critical point drying. Before investigation specimens were coated with coal and gold evaporation. Observation was made by Hitachi S-2360 N scanning microscope at 15 and 25 kV accelerating voltages.

Detection of nitrogen-fixing activity

The nitrogen-fixing activity was measured by acetylene reduction assay [12]. This was performed using one leaf of acclimatized srawberry per sample in glass reaction flask (30 ml Vitrotech bottles). Each treatment consisted of three to five replicates. The flasks had an atmosphere of 70% normal air and 30% acetylene. After 24-h incubation, the ethylene content was detected with Chrompack CP 9001 gas chromatograph using an Al_2O_3/KCl Plot column. Assays were conducted at room temperature. No attempt was made to quantify activity, only to show, qualitatively, that active nitrogenase was present.

Results

Establishment of strawberry-Azomonas/Azotobacter associations

For introduction of bacteria into the plant tissues, three possible ways were used and compared.

1.) inoculation of plant cell suspension with bacteria and incubation of the system
2.) inoculation and incubation of young primary leaves with bacterial cells
3.) introduction of bacteria directly into the leaf tissu and shoot apexes using the biolistic gun

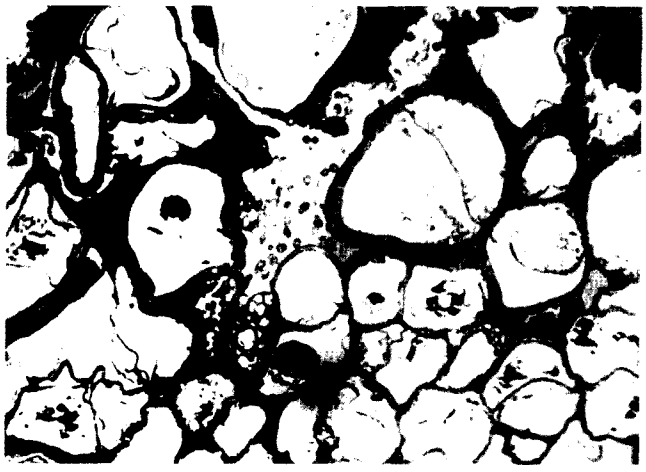
Inoculation of plant cell suspension with bacterial cells

From leaves of micropropagated plants we obtained callus then cell suspension wich contained unit cells and small cell aggregates. This cell suspension was mixed and cultivated with *Azotobacter* cells. On solid medium we obtained callus again wich already contained bacteria. While the inoculation procedure is rather easy whereas the incubation of the two partners during the *in vitro* phase, some criteia are neaded. It is important to emphasize that all media contained inorganic nitrogen source to promote plant regeneration from callus tissue. The incubation media contains maltose as carbon and energy source for the plant cells but not for *Azomonas.*

Using the above mentioned method it is possible to get *Azomonas* containing stawberry callus tissues. Location of the bacteria was detected by light and electron microscopy. Figure 1A, 1B show that calli contain a large

number of bacteria between the cells. Plant regeneration from these calli were not successful.

(A)



(B)

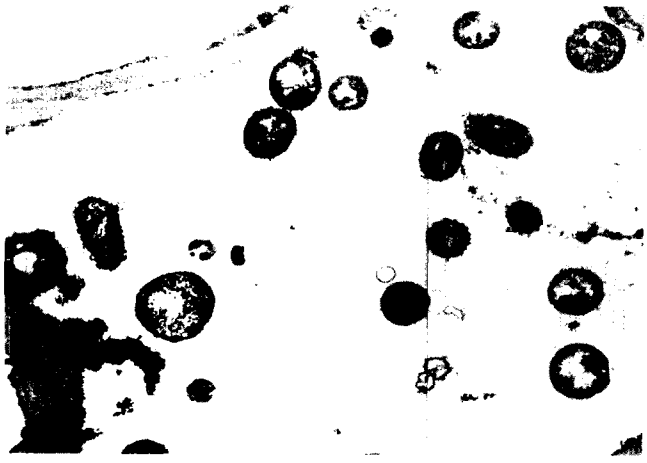


Figure 1. Micrographs of *Azomonas insignis* containing strawberry callus tissues. The samples were collected from callus tissues 14 d after the leaf inoculation with the nitrogen fixing bacteria. For the light micrograph (a), the semithin sections were stained by toluidine blue (1%). For the electron micrograph (b), the ultrathin sections were stained by uranyl and lead salts and observed by Hitachi 7100 transmission elctron microscope (X 3200). In both cases (a and b), $Azomonas cells are present in the intercellular spaces among the parenchymatic cells of the stawberry callus. The arrow = bacterium cells.

Inoculation and incubation of young primary leaves with bacterial cells

The young leaves of strawberry were co-cultured for overnight with the bacterium suspension (10^5-10^6 cell ml^{-1}) at 120 rpm agitation. The inoculated leaves were placed on callus inducing medium. During the callus formation,

Figure 2. Electron micrograph of $Azomonas insignis strawberry callus tissue. The ultrathin sections were stained by uranyl and lead salts and observed by Hitachi 7100 transmission electron microscope. (X3200). The $Azomonas cells are present in the intercellular spaces among the parenchymatic cells of the callus.

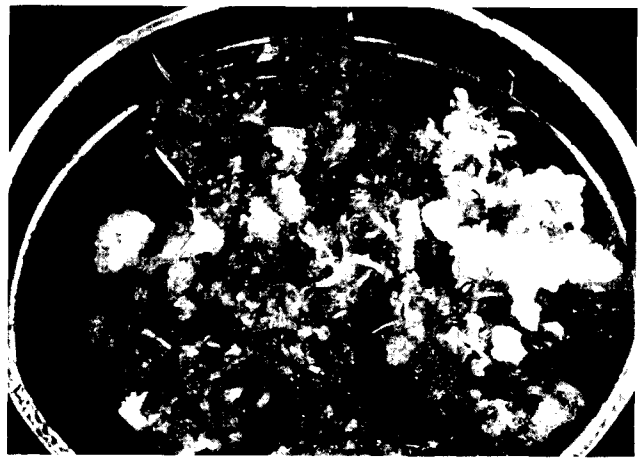


Figure 3. Regeneration process from the bacterium-containing srtawberry callus. Regeneration occurred 1 mo. after leaf inoculation with $Azomonas insignis on MS medium mineral salts + 555 M myo-inositol, 1.2M thiamine HCl, 4.4 M N6-benzyladenine (BA), 0.5 M indole-3- butyric acid (IBA), 0.3 M -naphthaleneacetic acid (NAA), 0.5 M 2,4-dichlorophenoxyacetic acid (2.4;D).

at the site of the injuries on the leaves, there is a random inclusion of the bacteria. Presence of bacteria was detected by light and electron microscopy (Figure 2). Introduction of bacterica from the callus tissues to the regenerated plants was also proved by microscopy(Figure 3). *Azomonas* cells were found in the intercellular spaces of both the petiole and the lamina (Figure 4). In the petiole they were located mostly under the epidermis and in the outer cell layers of the cortex, but some of them were found among the tracheary elements. In the lamina, bac-

teria were present in the spongy parenchyma.

Biolistic treatment of leaf inocules and shoot apexes

For direct and succesful transfer of the bacteria into the plant tissues, the particle bombardment method was adapted. This was the first use of living bacteria as microprojectile. Azotobacters were the bacterium partner in this case the lactose was used as carbon,-and energy sorence for plant tissues but not for the bacterium.

A week after bombardment, leaves were examined by microscopy and brownish spots were observed on the leaves by light microscope (Figure 5). Scanning electron micrographs cearly show the hit sites on the surface. There are holes of several cells in diameter, scattered on the leaves, which are probably caused by the tungsten aggregates (Figure 6) With larger magnification smaller penetration sites can also be seen on cell surfaces, torn by solitary microprojectiles. Examining the leaves two weeks later, we could observe that those bacteria which could not penetrate inside the tissues and remained outside on the leaf surface divided and multiplied to a larger amount of bacterial cells.

Bombarded leaves probably developed callus from

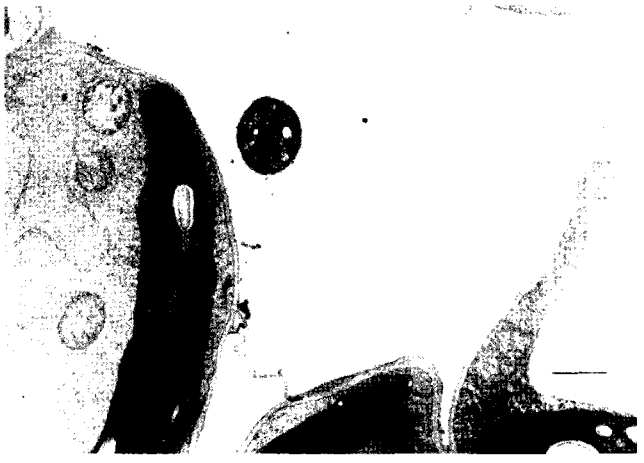


Figure 4. Electronmicrographs of $Azomonas insignis in the regenerated strawberry tissues (a = X6000, b = X5000). For the ultrathin sections, the samples were collected from the regenerated, 2-3 mm long leafy shoot tip primordia 28 d after the leaf inoculation with $Azomonas insignis. Bateria are located in the intercellular spaces among the chloroplast-containing cells.

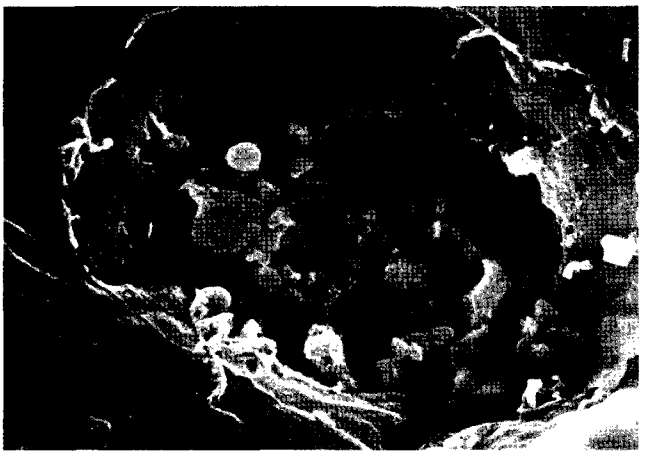


Figure 6. Scanning electronmicrograph of a deeper hit site among the epidermal cells with the delivered bacteria.

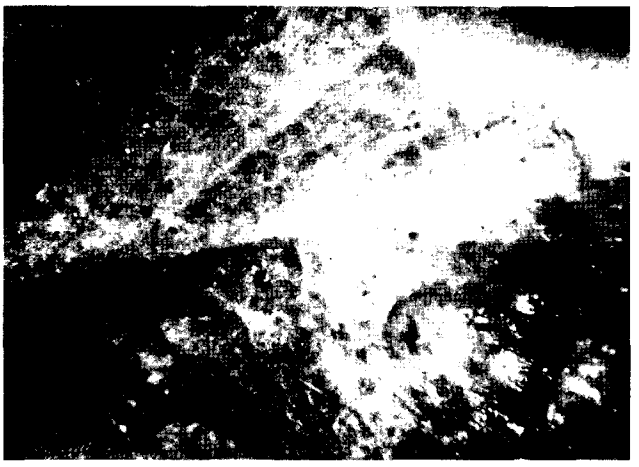


Figure 5. Leaf surface examined 1 week after the bombardment. The dark spots are the hit sites of the microprojectiles.

Figure 7. Callus development observed by canning electron microscope.

the injuries. Yellow coloured primary calli appeared 14-18 days after bombardment. The scanning electron micrographs show the early stage of the callus development (Figure 7). The presence of bacteria could be detected by transmission electron microscopy. The Figure 8 proves that Azotobacters are present in the intercellular spaces of the callus tissues. Division of bacterial cells had occured at their sites, demonstrating that conditions were suitable for growth and reproduction.

To enhance the safety and efficiency of the bacterium delivery and shorten the time after the biolostic treatment shoot apexes were use as target tissue. Continous division of the meristematic region ensures the spread of the bombarded bacteria in the forming organs and the whole plant.

The scanning electron micrograph demonstrate the situation 2 hours after the biolistic treatment. The tungsten particles caused injuries in the internal meristem tissue but that is able to regenerate in time and grow to a vigorous plantlet (Figure 9). A sufficient number of bacterial cells landed into this part of shoot apex. Azotobacters survived the impact, this is proved by the diving bacterium cell on Figure 10.

Figure 8. Electronmicrograph of bacteria among callus cells developed from the bombarded leaves.

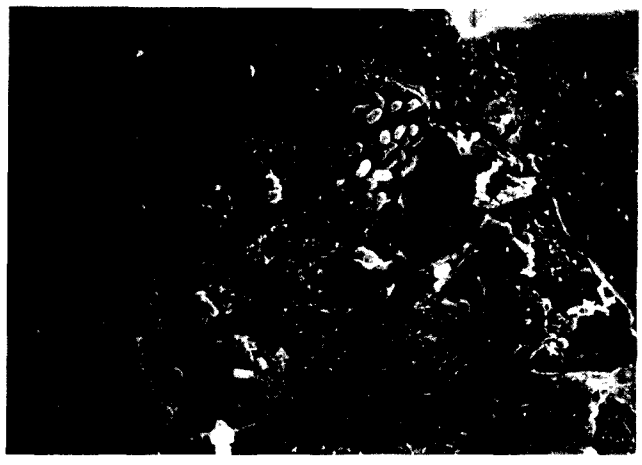


Figure 10. Injuries on the shoot apex surface, full of bacteria.

Figure 9. Bacterium bombardment of shoot apexes regenerated from strawberry callus. Saples were taken and fixed three hours after the treatment. The injuries are caused by the tunsten microprojectils.

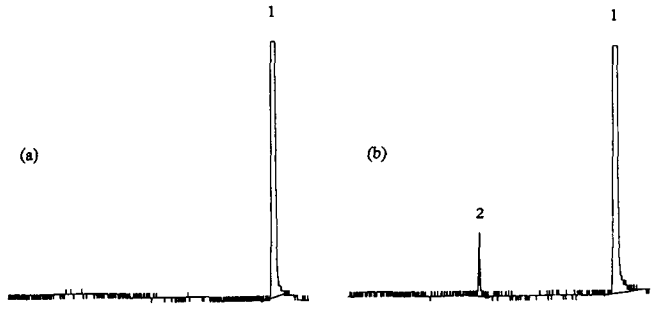


Figure 11. Acetylene reduction assay measured in control plant (a) and those containing bacteria (b). The youngest expanded leaves were collected from the acclimatized strawberries 1 mo after the plantation. The samples were incubated for 24 h in an atmosphere containing 30% acetylene. The nitrogen fixing ability can be detected by the ethylene forming from acetylene. The other (ethylene) peak on (b) shows the difference between the control and bacterium containing plant. 1 = acetylene, 2 = ethylene.

Acetylene reduction assay

In addition to morphological examination, the successful symbioses were functionally analyzed by measuring the nitrogenase activity. Compared to the control plants the bacterium-contaning strawberry leaves produced higher ethylene peak indicating the effective nitrogen fixation of those plants (Figure 11). Nitrogenase activity could be meansured in about 20% of the potted plants.

Discussion

Based on the algal models (10) it was possible to establish a stable association between the diazotrophic. *Azomonas insignis, Azotobacter vinelandii* and strawberry (*Fragaria x ananassa*) wich can be regarded as an endosymbiosis and exocytobiosis. The bacteria gained acces into the plant regenerated from callus via organogenesis. In contrast to somatic embriogenesis, organogenesis starts from a group of cells wich eusures a better possibility for bacterium to spead in the forming organs. Plant regeneration from callus was stimulated by nitrogen in the media which did not repress bacterial nitrogen fixation from air.

Applying the method of DNA transfer by particle bombardment, bacterium cells adhered to tungsten particles were shot into the target incoules: leaves and shoot tip meristems. The tungsten size (2-3 μm) was chosen according to the bacteriun size. This size is approximately duoble than that of generally used for gene delivery, but the damages caused in the target plant tissues are not more significant. The pressure of the accelerating nitrogen gas (30 bar) ensures the incorporation of bacterium cells into deeper layers of the plant tissues. Bacterial cells can tolerate this chinetic energy and the injured tissues are able to regenerate. This is proved by the spread of Azotobacters in the developing strawberry organs and tissues.

Particle bombardment seems to be an efficient method for establishing artificial nitrogen fixing associations as it ensures to introduce diazotropic bacteria with a high density into the target tissue. Theoretically, any higher plant can be inoculated by nitrogen fixing bacteria with this method. The fact that bacteria can gain acces to the newly forming tissues and organs is of great importance with the vegetatively propagated plants. This ensures the transfer of bacteria into the new generation.

Bacterial cells are located in the intercelllular spaces of the plant tissues which are important sites of the apoplastic transport and where both of the partners excrete essential compounds for each other. *Azonomas*, like Azotobacters in general, provides reduced nitrogen source manly as ammonia, amino acids, and vitamins but also exudes plant hormones (heteroauxins) [14, 20]. Plant cells may supply the bacteria with carbon and energy source, transforming the sugars presented in the media during the *in vitro* period and photosynthesizing leaves, in the *ex vitro* phase.

The basis of natural symbioses is the interdependence of partners. It is important to emphasize that all media contained an inorganic nitrogen source to promote regeneration. Therefore, only one-sided bacterial dependency was ensured by the sugar source during the introduction of the bacteria into the plant tissues. Maltose present in the media can be utilized directly by srawberry but not by the Azomonas [24]. In the case of the Azotobacter the incubation media containslactose insteal of maltose. Bacteria can use only those sugars as a carbon and energy source that are produced by plant cells and exreted into the intercelluar spaces [11, 15]. The interdependency is realized when bacterium-containing regererated plants are planted out in the soil because they then become autotrophic organisms. As it is hoped, the system at this time turns into a real symbiosis.

The main advantage of this method is that bacterium cells are present in all parts of the entire plant. This indicates the possibility of multiplication of bacteria in the regenerated plants. Because Azotobacters are aerobic organisms, in addition to the root system they can also function in the photosyntherically active leaves. Nitrogen fixation, meansured by the acetylene reduction assay, could be detected in about 20% of the potted plants. This rate is probably caused by the random introduction of bacterium cells into plant tissues.

Our artifical symbioses have both evolutional and practical significance. They provide model systems for the sudy of plant-microbe interactions and evolution but at the same time they may be helpful in the transfer of desired abilities into new host. The transfer of nif genes was implemented by the introduction of whole diazotrophic prokaryotes. In the case of higher plants, this type of transfer may be more advantageous than manipulation of relevant genes.

Acknowledgements

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