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Effects of Antibiotics on the Survival of *Agrobacterium tumefaciens* on Cowpea (*Vigna unguiculata* (L) Walp) Cultures.

O.O. Agarry,* O.B. Olorunfemi, Y.A. Babarinde and F.C. Adetuyi

Department of Microbiology, Federal University of Technology, P.M.B. 704. Akure. Nigeria.

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Transgenic plants of cowpea (V*igna unguiculata* (L) Walp) were generated using *Agrobacterium tumefaciens*. The antibiotics that eliminated the *Agrobacterium tumefaciens* totally were 200mg/l ampicillin and 500mg/l cefotaxime. The co-cultivated embryos muddled with *Agrobacterium* were cultured on different concentration of Carbenicillin, Augmentin, Ampicillin and Cefotaxime. The surviving shoots were transferred every ten days to ensure cleaning and contact with the medium. At each transfer the number of surviving shoot decreased due to shock of *Agrobacterium* contamination until it stabilized. Those plated on 200mg/l Ampicillin and Cefotaxime eventually produced new green healthier auxiliary shoots which could be transferred to the soil.

Keywords: Transgenic plants, Antibiotics, *Agrobacterium tumefaciens*.

Cowpea is a key staple food for many developing countries of the tropic zone. It supplies about 50% of the daily proteins requirements. The chemical composition of cowpea is similar to that of most edible legumes. It contains about 24% protein, 62% soluble carbohydrate and small amount of other nutrients (Ohlander,1977). Cowpea contain anti-physiological substances such as lectin and trypsin inhibitors as well as polyphenels or considered tannins, which have been receiving attention since they decrease protein digestibility and reduced protein quality. Physiological problems manifested during the vegetative stage of compact genotype has the most severe effect on seed yield, reductions of about 48% had been reported. (IITA,1982).

Although, wild *Vigna* species have high levels of resistance to specific insect pests. Most of these could not be a source of resistance in cowpea breeding because they were incompatible with cultivated cowpea, for example *Vigna vexillata* was identified as having high level of resistance to the legume pod borer (IITA, 1982). Studies on hybrid ovules revealed that hybrid embryos degenerated at the globular stage and that the percentage of fertilized ovules was only 15-20%, attempt to rescue embryos was not successful (Filipone, 1990). Due to failure in conventional approaches to cowpea improvement, including wide crosses and embryo rescue, biotechnology may be a useful tool in cowpea breeding.

However, applications of molecular genetic techniques mostly rely on efficient plant regeneration from *in vitro* cultures, successful plant regeneration has been reported in *Vigna unguiculata* (Jackai, 1992). Various chemical treatments have been used to stimulate DNA uptake by protoplasts. At present, polyethylene glycol (PEG) is the most common chemical treatment used to stimulate DNA uptake into protoplast (Neuhaus *et al.*, 1987). PEG increase the permeability of cell membranes, and has been used as an efficient protoplast fusion agent in somatic cell dilation of many plants and animals species. PEG concentration can affect protoplast

* To whom all correspondence should be addressed.
E-mail: oluagarry@yahoo.com

viability and gene transfer efficiency. The optimal concentration of PEG is 15-25%. If PEG concentration is too high, cell viability will decrease, and if too low, the gene transfer efficiency will decrease. (Neuhaus *et al*., 1987)

Agrobacterium tumefaciens, the causative agent of crown gall disease usually appeared in nature as tumours of root and stem of more than one hundred susceptible plant species. The natural gene transferring ability of *Agrobacterium tumefaciens* can be exploited to genetically modified plants. The transfer of DNA from *A. tumefaciens* to plant cells is a consequence of specific DNS-protein interactions between *Agrobacterium tumefaciens* and plants (Puont-Kaerlas *et al.,* 1990). A number of grain and pasture legumes are now amenable to gene transfer by genetic engineering using the Agrobacterium-mediated gene delivery system. These include, soybean (Hinchee *et al*., 1988), pea (Pounti-Kaerlas *et al*., 1990), and *Medicago sativa* (Deak *et al*., 1986). *Agrobacterium tumefaciens* mediated transformation of various pea explants has also been reported on axis and epicotyl segments (Fillipone and Lurguin, 1989). The co-culture period is usually two days. At the end of this period, explants are transferred into a medium containing antibiotics to kill the bacteria and to select transformed cells from untransformed ones. It is necessary to set up the appropriate level of antibiotics to select transformed cells without affecting the plant regeneration pathway and at the same time prevents the possibility of escape of untransformed plants. This study was carried out therefore to set a protocol for cowpea transformation, and controlling the problems posed by *Agrobacterium tumefaciens* on plants transformation using antibiotics.

MATERIALS AND METHODS

Test samples

Seeds used were obtained from the Biotechnology Research Unit of International Institute of Tropical Agriculture (IITA), Ibadan. *Agrobacterium* (AGL, PKLYX pe6 70-3n) genes which is virus resistant was supplied by John Innis Centre, UK, which came in a glycerol steek. The glycerol steek was streaked-out on a solid medium, and a loopful of the organism was picked and streaked on LB Agar medium containing selective antibiotics (50 mg/l of Kanamycin and 100 mg/l of rifampicin). This was incubated in a dark incubation room for 24 to 48 h. After the growth of *Agrobacterium* on solid medium, it was inoculated into LB broth medium which contained same antibiotics as in LB Agar medium. The culture was incubated aseptically at room temperature over a shaker at low speed for 24 and 48 h

Cowpea seed sterilization and embryos rescue (wounding)

Cowpea seeds were sterilized overnight with 2g of sodium hypochloride per litre and 1ml/l of Tween-20 mixed together in distilled water. The embryos of some seeds were removed after rinsing and the first two leaves present on the embryos were cut with sterile scalpel blade. This served as the wounding site or route of entry for *Agrobacterium*, thus decapitation was done.

Co-cultivation with *Agrobacterium* broth culture

Agrobacterium cells in LB broth culture were spun down using low speed, and the supernatant decanted. The cells were later re-suspended in equivalent amount of MS broth medium. The wounded cowpea embryos were co-cultivated in this broth culture for 2 h.

Vacuum Interaction

Vacuum interaction was carried out for 30min. using Gene gun to inculcate *Agrobacterium* into the plant via the wounded site.

Co-cultivation on growth medium

The broth culture was decanted, embryos dried on sterile tissue paper under laminar air flow hood and then planted on MS agar medium. This medium did not contain any antibiotic against *Agrobacterium*, this is because, the co-cultivation of *Agrobacterium* will continue for about 7 to 10 days. The medium was incubated at ambient temperature and in the dark for 5 days, before the petri dishes were brought under light for another 7 days. The *Agrobacterium* grew round each of the embryos which actually indicated proper infection. Rooted plantlets were cut off from the bottom to allow proper contact with the medium and to allow efficient selection of the plants. Sub-culturing was done every 10 days to ensure that the plants did not run out of supply of nutrient

provided by the medium.

Washing in antibiotics solution

The decapitated explants due for selection medium were soaked in solution of Ampicillin and Cefotaxime at concentration of 200mg/l and 500mg/l and put on shaker overnight. The explants were then rinsed 3 times with sterile distilled water under the laminar air flow.

The *Agrobacterium tumefaciens* used in this work grew very well on LB agar and LB broth medium (50mg/l of kanamycin and 100mg/l rifampicin) indicating that the bacteria contained a kanamycin-resistance plasmid (Table 1). The vacuum infiltration with the gene gun helps to force *Agrobacterium* into the explants. After transformation of embryos, the *Agrobacterium* was still noticed around each embryo on co-culture

RESULTS AND DISCUSSION

Table 1. The number of explant used for the experiment

Medium	No of explant	No contaminated	Death due to stress	No of explant recovered
Inoculated LB Broth	150 + or -2.00	-	-	150 + or -2.00
Ms Growth medium	150 + or -2.00	20+ or -1.00	15 + or -1.00	115 + or -1.00
Selection medium	115 + or -1.00	10 + or -1.50	45 + or -1.5	7

medium which means complete infection of embryos/explants. The number of explants recovered reduced as they passed through ranges of medium. Table 1 shows different types of medium used for the cowpea explants, quantity contaminated, and explants recovered. The rate of plant growth was not affected by the antibiotics used for the *Agrobacterium* control. But the explants were affected by the *Agrobacterium* which grew round the explants and prevented them to feed from the media. The numbers of plantlets on selection with various concentrations of antibiotics were observed from day 2 to day 10. The rate of *Agrobacterium* growth round the explants which lack sensitive antibiotics and concentration enough to get rid of *Agrobacterium* were abnormal, stunted and the colour turned brown (Plate 1). The reason is that *Agrobacterium* grew round the area that submerged in the medium and deprive the plant of normal nutrition from the medium. From day 3, the growth of *Agrobacterium* were noticed round the plants and later spread through the plates. When the antibiotics were able to seize the growth of *Agrobacterium*, the plants were observed to be cleaned and healthy green and have normal growth as expected on the media. The fair growth of plant and *Agrobacterium* were noticed when high concentration of cefotaxime was used with carbenicillin. The fair growth is due to the fact that the antibiotics were slightly sensitive. There were no *Agrobacterium* growth with high concentration of cefotaxime and ampicillin, the plants were clean and green (B and C). When the explants were washed in solution of Ampicillin and cefotaxime overnight and rinsed 3 times with sterile distilled water and plotted on sterile tissue paper, no growth of *Agrobacterium* was observed in subsequent culture. The successful plant (D) were rooted and transferred to peat pellet and nurtured for further analysis.

It was observed that some plants were lost because of difficulty encountered in controlling *Agrobacterium*. The colour of the medium did not change indicating that little or no metabolite was released into the medium which means the medium really support the growth of plantlets. However, the *Agrobacterium tumefaciens* were observed to have grown round the plantlets within first week of the experiment. Filipone *et al.* (1992) used 250mg/l of carbenicillin to control *Agrobacterium* in plant transformation. The concentration of antibiotics and the most sensitive antibiotic use for a

Plate 1. Number of plantlets on selection with various concentration of antibiotics (observed from day 2 to day 10)

Control:	Plate without antibiotics
A:	Plate containing 50 mg/1 Amp. + 250 mg/ 1 Carb.
B:	Plate containing 100 mg/1 Aug. + 50 mg/ 1 Amp.
C:	Plate containing 500 mg/1 Cefotaxime + 100 mg/ 1 Carb.
D:	Plate containing 200 mg/1 Amp. + 500 mg/ 1 Cefotaxime

particular strain was compounded by the construction of plasmids with different antibiotics as selective agent. The *Agrobacterium* which has carbenicillin as selection in its preparation would not be eliminated, this is because it has carbenicillin resistance gene. It was observed that Ampicillin and cefotaxime were able to eliminate *Agrobacterium* because, gene which uses them as selection are not really available and they succumbed easily to the antibiotics within few days of culturing. The selection medium should get rid of untransformed plantlets if properly selected with right dose (kanamycin and ppt). When *Agrobacterium* was properly eliminated, it can as well kill transplanted plants by softening the plant tissues. The few explants derived were obtained when the right antibiotics were used at correct concentration of ampicillin and cefotaxime. The inefficiency of antibiotics to curb the excessive growth of *Agrobacterium* causes abnormal growth of the plants in the sense that there is interference of the *Agrobacterium* between the plants and medium used which invariably prevented the absorption of medium nutrient, resulting in starvation, stunted growth and death.

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