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Induced Herbicide Resistance in Certain Food Legumes Using *In Vitro* Techniques

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Abstract: Recently new approaches were developed to produce tissue and cell culture techniques capable of regeneration in to fertile plants in recalcitrant Legume crops by via either Organogenesis or Embryogenesis. The suspension culture system facilitates an experimental approach with a large variety of objectives for crop modification cellular selection and transformation. The importance of plant cell and tissue in applied and fundamental research has out lined by many workers. Though Legumes have been extensively used in plant cell and tissue culture systems regeneration of whole plants from *in vitro* experiments not amicable like other systems. The growth of excised plant tissues on defined medium should provide a relatively precise measurement of the effect of growth regulating substances as compared with the evalution of their influence on whole plant. This approach is tested in the food Legumes by comparing auxin and cytokinin requirements on callus tissue derived from different genotypes. The objective was to identify normal and plant types with distinct auxin and cytokinin response to be used in studies of genetic regulation of hormonal function and metabolism.

Keywords: Cowpea (Pusa), Chickpea (Annigiri), Soybean (Jack) and Cluster Bean (Pusa Navabhar)

1. Introduction

Grain legumes, commonly known as pulses, form an integral part of the vegetarian diet in the Indian sub-continent. Besides being a rich source of protein, they maintain soil fertility through biological nitrogen fixation by bacteria prevalent in their root nodules and also conserve and improve physical properties of soil by virtue of their deep and well spread root system. Pulses are also used as green fodder for animals. Our country can take a rightful pride in attaining self sufficiency in the production of food grains but it has miserably failed in case of pulses, for which we are even today dependent on import to a large extent. The food grains production has crossed 200 million tonnes mark, yet crop imbalances are still there. This is because certain crops like pulses have not experienced the impact of green revolution. Pulses occupy a larger part of the area (22-24 million hectares) under cultivation in India but their production is low. The production was lower at 13.4 million tonnes in (2004-2005) as against the production of 14.9 million tonnes in 2003-04. The availability of pulses in the international market is limited. A solution to the problem of the declining per capita availability has, therefore, to come from a rapid improvement in indigenous production levels.

Indian population is growing at a rapid rate, and hence the stagnation in pulse production has meant a severe decline in the per capita pulse availability, a matter of serious concern in a country where pulses are the major source of protein for most of the people. The non-availability of high yielding varieties is a major constraint in achieving higher productivity of pulses. Non-synchronous maturity, long duration and flower drop are other problems associated with the varieties of major pulses. Chickpea or Bengal gram (*Cicer arietinum* L.) is an annual grain legume or pulse crop that is used for human consumption in India.

Most farmers in developing countries are small landholders, and they usually grow legumes after cereal crops such as rice, corn and sorghum. Post-harvest handling, transportation and storage are the most important factors to consider in producing quality dry legumes. Inefficient post-harvest handling of legumes in developing countries has affected grain quality. Most farmers in developing countries dry their mature beans under the sun. Several locally made threshing, shelling and grading machines have been introduced by local merchants. The traditional processed soybean food products known in Southeast Asia can be classified into two categories: 1) non-fermented and 2) fermented. The former includes soybean milk, tofu or soybean curd and yuba. Soybean milk can be prepared at home by grinding whole soybean with water and straining to make rich, creamy milk-like liquid called "soy milk". The product has a unique, beany flavor, which is a well-accepted beverage in Southeast Asian countries. Tofu is closely associated with soy milk. Yuba is a sheet-like coagulant formed on the surface of warm soy milk as it cools. The lifted sheet of soybean contains emulsified oil from soy milk and has high protein with a delicate flavor. The production is still at the cottage scale and industry level technology is yet to be developed. Fermented soybean products include soy sauce and soy paste. Both soy sauce and soy paste products have to be processed through the fermentation step of "koji" production. Most fermented soy products are processed into both soy sauce and soy paste in the ratio 70:30, 60:40 and 50:50 depending on the market requirements. Other fermented soybean products are fermented soybean, fermented white soybean and imitation fried pork rind. Peanut is prepared for direct consumption by boiling, drying, roasting and confectioning. Peanut bar and coated roasted peanut products are processed by using modern equipment. The products are packed in good quality packaging to control moisture and air to prolong shelf life. About 73 percent of mung bean production is used for local consumption.

The largest processing industry is transparent noodle production and starch extraction, which accounts for about 20 percent. Transparent noodles are processed from mung bean starch. Bean sprouts are produced from mung beans and black grams. The product is produced for fresh market and for daily consumption. Bean sprouts are processed on a home or cottage scale, using traditional technology. The soybean industry has introduced a number of new infrastructure and technologies which have, and will continue to have, significant impact on farming methods, bulk commodity storage, handling and distribution. Direct utilization of soybean in the food industry includes full fat soyflour for baking, soy-based beverages, and snack foods. Texturized vegetable protein (TVP) made from soyflour using single and twin screw cooker extrusion is available in dry forms. Peanut processing technology has been developed similar to soybean. Peanut flour is obtained from oil extraction process. Various snacks developed from peanut bases are packed in attractive packaging. Peanut butter processing is quite a large industry. A mung bean cracking machine, a grinder which separates liquid and solid, a starch separator, a starch mixer and a noodle machine have been developed for making transparent noodles. At present, modern biotechnology to produce legumes with herbicide resistance has increased public awareness on biosafety and food safety. Consumers around the world have different views towards the technology. Labeling has become a major issue. However, specific method to determine genetically modified organisms (GMO) and genetically modified foods (GMF) requires more scientific information to make labeling possible. Grains would be the fastest track to increase production capacity to meet the world population needs. This paper reports the results obtained from the study of auxins response to shoot multiplication, production of callus, morphogenesis and herbicide tolerance in certain food Legumes.

2. Materials and Methods

The certified seed material of four important food legumes of Cowpea (Pusa), Chickpea (Annigiri), Soybean (Jack) and Cluster bean (Pusa Navabhar) were obtained from Acharya N. G. Ranga Agriculture University Research Station, Warangal (A. P). Aseptic seedlings were raised by serface sterilization of seeds with 70% ethanol for about 2min followed by 0.1% aqueous mercuric chloride solution for 10 min. The seeds were thoroughly washed with sterile water and then place aseptically on MS or B5 medium containing sucrose and 0.8% agar-agar. All the media were adjusted to pH 5.8 before autoclaving at 1.04 kg for 15 min. The seeds were allowed to germinate at 25+2°C under 16h photoperiod and light intensity of 2000lux. Cotyledons from 3day old seedling were excised and cultured on MS, B5 and MSD medium supplemented with different concentrations of BA (0.1-2.0 mg/l), Ads (0.1-2 mg/l) and KN (0.1-2 mg/l) to investigate the morphogenetic potentiality and regeneration by production of multiple shoots. Depending up on the crop and cultivar specificity various concentration and combinations of plant growth regulators includes 2, 4-D, BA, Kinetin etc., for initiation of unorganized static cultures and differentiation studies.

After subsequent establishment of callus cultures, 0.5gm of tissue was transferred to a liquid M. S medium for growth and maintenance of suspension cultures to make selection of cell line experiments. In the case of chickpea

and cluster bean the static cultures were used for selection studies on agar solidified Petri dishes containing herbicide in the medium. Because of very poor response on the liquid medium for growth of suspension cultures. Parameters like, inhibition of growth studies, plating efficiency and growth index were studied at different growth interval and transferred them to fresh nutrients medium. To investigate the morphogenetic studies and multiple shoot induction from seedling explants, we have used mainly cotyledons and cotyledonary nodal explants inoculating them on to the MS and B_5 medium. For induction of somatic embryos in soybean, immature pods of first yield, which contains immature embryos, have been utilized and transferred then on to MSD 20 and MSD 40 medium. For the last one and half decade experimental studies were carried out under *in vitro* and in vivo interaction of pesticides and herbicide and their tolerance at cellular level using tissue culture system. During the course of these investigations certain cell lines selected against Atrazine, Anthio, Monocrptophos and Glyphosate. Glyphoste is a systamic, non-selective herbicide, used for induction of genetic tolerance in soybean embryogenic cell suspension cultures on FG liquid medium.

3. Results and Discussion

The establishment of an efficient plant tissue culture is a basic step in static culture system to study the morphogenetic ability. For the purpose of unorganized static cultures, which are considered to be first and foremost step for all *in vitro* experimental investigations, we used four different species of food legumes, *Vigna*, *Cicer, Cymopsis and Glycine* of local cultivars. In cluster bean various aseptically grown one week seedling explants were cultured on B5 medium [1 and 2] with 1.50 mg/l 2, 4-D and 0.50 mg/l kinetin. Root tips and immature leaf explants were poorly responded for callus initiation with brownish clumps of proliferated tissue wounded sites. Due to high secretion of phenolic compounds on to the medium callus cultures were turned to brownish and to the callus and low frequency of callus initiation [3].

For induction of callus cultures, the seedling explants are found to be very efficient in producing friable whitish high morphogenetic callus in *Vigna sinensis*. Somatic Embryogenesis and multiple shoot induction were made from *Vigna* cotyledonary explants with MS medium 1.5 mg/L BA where highest shoots (6.2) forming ability was observed and 69% of differentiation observed in the cultures (Table 2).

Table 1. *Morphogenetic response of different explants of Grain legumes on MS/B5 medium with different concentrations of 2, 4-D.*

Plant Species	Media / 2, 4-D	Explants used	% of Cultures responding	Nature of culture	Selection agents applied (ppm)
V. mungo	MS/2.5mg/L	Cot	69	Embryogenic Callus	Atrazine 90ppm
,,	,,	HC	84	,,	,,
,,	,,	IL	54	,,	,,
,,	,,	RT	50	,,	,,
C. arietinum	B5/2.25mg/L	EC	79	Organogenic Callus	MCP 60ppm
,,	,,	HC	57	,,	,,
,,	,,	IL	42	,,	,,
,,	,,	RT	59	,,	,,
Cyamopsis	B5/2.0mg/L	Cot	60	Organogenic Callus	Anthio 40ppm
,,	,,	HC	84	,,	,,
,,	,,	IL	50	,,	,,
,,	,,	RT	54	,,	,,
G. maxo	B5/3.0mg/L	Cot	67	Embryogenic Callus	Glyphostate 20mM
,,	,,	HC	56	,,	,,
,,	,,	IL	52	,,	,,
,,	,,	RT	50	,,	,,

Cot: Cotyledons. HT: Hypocotyledons. EC: Epicotyl. IL: Immature leaf. RT: Root tip MCP: Monocrotophos.

Using unorganized static culture system which was derived from seedling explants has been utilized for genetic modification of some important food Legumes including beans etc., [4 and 5]. Very recently we could able to establish the suspension culture system on a liquid MS medium and studies the influence of Glyphosate on gene amplification. In the selected cell lines against 10 mM of Glyphosate increased level of the target gene product EPSP synthase enzyme was observed in *Cicer arietinum* due to amplification of DNA [6].

The clonal multiplication of cotyledonary explants by induction of multiple shoots facilitates the *in vitro* genetic transformation and transgenic plant production of Cowpea. An unorganized friable static cultures of *Vigna sinensis* were exposed to Atazine stress and isolated resistant cultures at 90 ppm conc. [7]. In glyphosate resistant carrot cell suspension cultures the increased EPSP synthase activity and amplification of the target DNA. Confers the herbicide resistance [8]. In chick pea the static cultures have been established on higher levels of auxin (3.25 mg/L 2, 4-D) and 2.0 mg/L of NAA on B5 medium. In cowpea direct shoot bud formation was observed in low frequency while inoculating the cotyledonary explants on the same medium with BAP.

High frequencies of callus initiation (84.50) with highest growth index (11.4) were observed in hypocotyl explants followed by cotyledons where growth index was 8.8 and percentage (60.35). In this case endogenous level of growth regulators were optimized with external supplementation in the medium responsible for the cell proliferation. The friable

callus was sub cultured on the same medium for selection against Anthio. This organo phosperous pesticide was able to induce tolerance at 60 ppm concentration on sensitive cluster bean static cultures and further using this we have selected Anthio resistant calli clones in *Cyamopsis tetragonoloba* (L.) Taub. These results were in conformity with other experimental studies on legumes with different species [9].

Cluster bean is a recalcitrant species among the food Legumes, we have made several attempts to induce somatic embryogenesis and *in vitro* shoot bud induction in the PNB cultivar. This cultivar is not amicable to tissue culture experiments, but few reports are available in current literature about morphogenetic ability of protoplast isolated from cotyledons of *Cymopsis*. We performed several experiments related to the *in vitro* studies of cluster bean and made documentation of several aspects related to tissue culture and morphogenesis [10, 3 and 11]

Table 2. *Effect of BAP/Kn/AD on induction of multiple shoots from Cotyledonary explants on MS/ B5 media of Grain Legumes.*

Plant Species	Media Differentiation	PGR	Hormone conc (mg/L)	% of cultures responding	Average no of shoots / explants (S.E.)*
V. mungo	MS	BAP	0.1	39	2.4 ± 0.21
,,	MS	BAP	0.5	57	4.8 ± 0.32
,,	MS	BAP	1.0	71	6.2 ± 0.40
Cicer	B5	BAP	0.1	36	3.6 ± 0.43
,,	B5	BAP	0.5	52	5.2 ± 0.30
,,	B5	BAP	1.0	58	5.0 ± 0.42
Cymopsis	B5	Kn	0.1	34	1.3 ± 0.32
,,	B5	Kn	0.5	37	2.7 ± 0.56
,,	B5	Kn	1.0	38	2.9 ± 0.26
Glycine	MS	AD	0.1	70	4.0 ± 0.30
,,	MS	AD	0.5	64	7.2 ± 0.03
,,	MS	AD	1.0	62	6.5 ± 0.73

*S. E. Standard Error

Though tissue culture and morphogenetic aspects of several soybean cultivars were well documented and no potential system for regeneration of complete plant lets is achieved so far. We have tried with immature pods containing embryos for induction of somatic embryos on MSD-40. Induced somatic embryos were separated proliferated on MSD-20, and maintained embryogenic cultures on FG with 1.81 uM of 2, 4-D and 6.0mg/L glycine. Selected somatic Embryos germinate on MS medium without any growth regulators produced plant let for acclimatization (Ramulu. 1997). The cell lines established with SB-P line were made step wise manner to induce Glyphosate resistance on MX 1.81 uM liquid medium. The selected SB-P cell line has increased the target Enzyme EPSPS folds that of wild type of cell lines. Under *in vitro* conditions Atrazine induced tolerant shoots were regenerated and isolated in soybean showed resistance to herbicide in field conditions. The excised cotyledons from 3 days old seedling of four Legume sps cultured on MS, B5 and MS medium supplemented with BA, Ads and Kinetin to investigate their morphogenetic behavior in production of direct multiple shoots and also from callus cultures and percentage of differentiation. Maximum number of shoots per explants 7.2+06 shoots / explants with 59% of differentiation was observed in *Glycine max* fallowed by 6.2+0.40 shoots / explants shoots with 69% differentiation in *Vigna* with Adenine sulphate 0.5 mg/l. Reduced number of shoots 2.9+0.26 shoots / explants with poor differentiation abilities 16% from cotyledon explants were observed in *Cicer* and *Cyamopisis* a recalcitrant species due to presence of low endogenous Plant Growth regulating Substances.

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