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An Evaluation upon Pigeonpea Improvement and Its Utilization: A Case Study of Rapid and Highly Competent Regeneration

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Abstract – Pigeon pea (Cajanus cajan (L) Millsp.) is an important multipurpose grain legume that is a good source of protein for populations living in the semi-arid tropics. Being a crop that is cultivated under rain-fed agricultural system, its production is threatened by several biotic and abiotic stresses. Attempts to address these problems through conventional breeding have achieved partial success due to narrow genetic variability among the cultivated species. In addition, breeding incompatibility problems associated with wild species warrant exploration of alternative approaches like gene transfer to introduce desirable traits. Development of in vitro regeneration protocols amenable to genetic transformation offer an attractive opportunity for improvement of pigeon pea.

Pigeon pea is legume crop play a crucial role as source of dietary protein in diet, growing extensively in the rainfed and dryland spots of India and worldwide. Plant tissue regenerate through in-vitro system attempting organogenesis as well as embryogenesis pathway, which are in support of unfamiliar genes assimilation targeted for development of transgenic plants. Present study was undertaken to investigate the most appropriate explant type in Pigeon pea regeneration by virtue of in vitro culture system. Genotype Durga (NTL-30) was breed and used as principal material for regeneration studies.

Pigeonpea (Cajanus cajan) is mainly a tropical crop which is cultivated with the cereal grains such as maize, millet and sorghum etc. Pigeonpea as a valuable cover crop grown for food (dry or green seeds), feed (seed, leaves and young branches), firewood, medicine, fencing, roofing, shade and to make baskets. Inspite of large land covered for cultivation of pigeonpea there is a wide demand–supply gap as its production is constrained by various biotic and abiotic stresses. So attempts for development of an efficient in vitro regeneration protocol are made for conservation of this important legume. This review brings light to various culture conditions, explants and hormonal combinations to develop efficient in vitro regeneration protocol in pigeonpea.

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INTRODUCTION

Pigeon pea (*Cajanus cajan* (L) Millsp.) is a drought tolerant pulse legume mainly grown for its grain in the semi-arid tropics of many developing. The Indian sub-continent, central America, and eastern and southern Africa are the major pigeon pea producing regions in the world. It is produced as a vegetable or as an export grain crop in the east and south African countries (Shiferaw *et al.,* 2008). In India, it is the third most important legume crop after beans and cowpeas, and is one of the fastest growing cash crops (Mergeai *et al.,* 2001).

Plant tissue culture is a technique of *in vitro* cultivation of plant cells, tissues and organs under defined physical and chemical conditions and controlled environment (Loyola-Vargas, 2008). Two of the basic pathways used for tissue culture of different plant species are organogenesis and somatic embryogenesis. Organogenesis involves regeneration of adventitious organs directly (without callus) or indirectly (with an intervening callus phase) from the explant. Somatic embryogenesis is the development of embryos from somatic cells either with an intervening callus phase (indirect somatic embryogenesis) or without an intervening callus phase (direct somatic embryogenesis).

Indirect regeneration often results in somaclonal variation making the strategy less desirable for large scale clonal propagation. Therefore, direct regeneration without callus phase is a reliable method for production of identical plants (Kharabian and Darabi, 2005). Successful *in vitro* regeneration depends on the control of morphogenesis which is influenced by the genotype, type of explant,

composition of nutrient medium, hormone and culture environment.

Pigeon pea widely well-known as red gram also locally "tura" in India, it is most significant food legume having crucial major grain legumes of the semi-arid tropics. It is grown commercially throughout the globe and cultivated in about fifty countries of Asia, Africa, and America for food, fodder, fuel, soil conservation, and green manure [Krishna G *et al*. 2011]. The productivity of pigeon pea are introverted by numerous diseases, including sterility mosaic, fusarium wilt, Phytophthora blight, Alternaria blight and major insect pest *Helicoverpa armigera*. Biotechnological application such as genetic transformation headed for superior pest resistance put forward opportunities for rapid improvement of pigeon pea. However the accessibility of an *in vitro* regeneration method is a prerequisite for effective plant regeneration. Regeneration through *in vitro* culture for exploitation of plant cell totipotency shows organogenesis and regeneration of shoot buds from various explants type of pigeon pea has been reported earlier by [Shrinivasan T *et.al* 2004]. Regeneration of pigeon pea via organogenesis has been reported with pre-existing meristem like apical meristem. Cotyledonary node. Embryonic axis [Krishna G *et al*.

2010].

The above published literatures suggest that addition of cytokinines in regeneration medium could be the underpinning for pigeon pea shoot bud discrimination [Dayal S *et al*. 2003]. Silver nitrate as an additive in medium promote plant growth regulation and morphogenesis in recalcitrant crop such as pigeon pea by means of participating silver ions in the form of nitrates as primary role and influencing somatic embryogenesis. Efficient shoot and root development which is the primary prerequisites for successful genetic transformation, reported earliear [Bais HP et *al*. 2000]. Hence forth the aim of the present research to fill up this unfilledness of shoots regeneration, proliferation and *in vitro* plantlets development. Pigeon pea regeneration through *in vitro* by addition of hormonal combination reported is rapid, Effortless and efficient for regeneration of cotyledornary node, embryonic axis and immature zygotic embryo (Scutellum) explants.

Pigeon pea (*Cajanus cajan* (L.) Millspaugh.) is one of the major grain legume crops in the tropical and subtropical regions of the world. India is the primary centre of origin and diversification for pigeon pea. It is also cultivated in Kenya, Uganda, Malawi, China, Myanmar and Nepal. It is an important source of protein and vitamin B. Pigeon pea seeds have 20-22% protein and are used as green peas, whole grain or split peas. Its seed husks and leaves are used as nutritious animal feed, while the stem is used as fuel and also for making baskets, thatching, fencing and huts. Pigeon pea fixes nitrogen in the soil and also reduces soil erosion. The species is diploid (2n=2x=22) with a genome size of 858 Mbp. It is a hardy, widely adapted and drought tolerant crop with a large temporal variation (97-299 days) for grain maturity. These traits allow its cultivation in a wide range of environments and different cropping systems. Globally Pigeon pea is cultivated on 4.64 mha with an annual production of 3.43 mt. The average productivity of 780 kg ha^{-1} (http:// faostat.fao.org/2010) indicates further need for improving its genetic potential. India is the largest Pigeon pea growing country in the world, accounting for 3.53 mha area with the production of 2.51 mt (http:// faostat.fao.org/2010) followed by Myanmar (0.58 mha), Kenya (0.17 mha), Malwai (0.12 mha), Tanzania (0.09 mha), Uganda (0.08 mha) and Nepal (0.03 mha) (http://faostat.fao.org/2010). The relatively low crop yields may be attributed to a lack of genetically superior varieties, low use of gene bank collections, poor crop husbandry and exposure to several biotic (diseases and insect pests) and abiotic (drought, salinity and water logging) stresses. Plant genetic resources are an invaluable source of genes and gene complexes for yield and several biotic and abiotic factors and provide raw materials for further genetic improvement. Therefore, the collection of Pigeon pea germplasm and its proper characterization and evaluation, conservation and utilization in improvement programmes assume great significance especially in view of climate change.

As compared to other Pigeon pea growing countries, the research and development activities in India are extensive with the first scientific Pigeon pea breeding effort initiated by Shaw in 1933, who studied morphological and agronomic traits of 86 elite indigenous Pigeon pea germplasm accessions. Some of the accessions were found to have high level of resistance to *Fusarium* wilt. Identical efforts were also made by, who reported some agronomically superior, early and late maturing high yielding lines of Pigeon pea. Considering the high significance of Pigeon pea in India, the Indian Council of Agricultural Research (ICAR) started an All India Co-ordinated Pigeon pea Improvement Project in 1965. Under its umbrella, genetic improvement programmes were started simultaneously at 30 research centres located in various agro-climatic zones of the country. The prime objectives of this programme were to collect Pigeon pea germplasm, identify stable sources of resistance to various diseases and insects and develop high yielding varieties in different maturity groups. Further, plant exploration and collection programme was initiated in a systematic manner with the establishment of a central agency for this purpose i.e. National Bureau of Plant Genetic Resources (NBPGR) Pusa New Delhi, India. During 1960-70, special efforts were made under the collaborative scheme between ICAR and the USDA to collect Pigeon pea germplasm from different parts of the country.

LITERATURE REVIEW

Cultivation of pigeon pea -

Pigeon pea cultivation goes back to at least 3000 years, originating from India from where it was introduced to east Africa. Pigeon peas are widely cultivated in all tropical and semi-tropical regions. Improved long (9 months), medium (6 months) and short (4 months) duration pigeon pea cultivars were developed and released in Kenya by the University of Nairobi (UoN), Kenya Agricultural Research Institute (KARI) and the International Crop Research Institute for Semi-Arid Tropics (ICRISAT). In Kenya, farmers predominantly grow long duration varieties that take up to 11 months to mature in the field; these late maturing genotypes produce rather low yields of between 300 to 500 kg per ha (Gwata and Shimelis, 2013). In addition, reports by FAOSTAT (2010) indicate that average pigeon pea production in Kenya is estimated to be 430 kg/ha.

Economic importance -

Pigeon peas is consumed as a food crop (dried peas, flour, or green vegetable peas) rich in mineral nutrients and it is also an excellent fodder because of its high nutritional value. Peas are nutritionally significant, as they contain high levels of protein (17 to 28 % protein) and they also contain important amino acids such as methionine, lysine and tryptophan hence a key food supplement for resource poor farmers who consume mainly low protein cereals and root crops (Prabakharan *et al.,* 2011). In combination with cereals such as maize, pigeon peas make a well balanced human food. Peas are also a source of income for small scale farmers in the arid and semi-arid lands.

Agronomy -

Pigeon pea is a hardy crop and can be grown on a wide range of soils ranging from sandy loam to clay loam and even on marginal soils. It thrives well on well drained black cotton and red laterite soils with pH 5-7. It is rarely found above altitudes of 2000 m above sea level. The optimum temperatures for pigeon peas cultivation ranges from 25 to 38°C however it can tolerate temperatures of up to 45°C if the soil has adequate moisture and fertility. On the other hand it does not tolerate low temperatures and frost conditions. It is also sensitive to high salinity water logging and does not tolerate shallow soil (Choudhary *et al*., 2011).

Pigeon pea growth is optimum under rainfall conditions of between 600-1000 mm/year, whereas on deep, well-structured soil it will grow well where rainfall is of between 250 to 370 mm/year. They have a deep root system enabling them to grow well in the dry areas. The bacterium *Rhizobium* that lives in the root nodules of the pigeon peas is able to fix nitrogen. Because of their capability to fix nitrogen they are important crop for green manure. After incorporation they can provide up to 40 kg nitrogen per hectare and hence improve soil fertility. Because of its extensive root system, they are able to take up nutrients and water from lower subsoil layers. Therefore, when intercropped they hardly compete with companion crops. This crop therefore grows and yields well under conditions of low rainfall and poor soils and apart from being a source of food the woody stems are used as source of household fuel, thatch and fencing for subsistence farmers.

***In vitro* culture of pigeon peas -**

Plant tissue culture is the growth of microbe free plant material in a controlled aseptic environment such as a sterilized nutrient medium in test tubes. It involves culture of plant parts, tissues and organs and is based on the fact that all plant cells retain their ability to use all their genes to develop into any tissue or a whole plant (Singh, 2003); commonly referred to as totipotency. Through micropropagation rapid multiplication of stock plant material to produce a large number of plants can be achieved, including those plants that do not produce seeds. Micropropagation is used to multiply novel plants, such as transgenics or plants bred through conventional methods.

Callus culture and somatic embryogenesis -

Callus induction has been achieved from epicotyls and cotyledon explants of pigeon pea using different concentrations and combinations of IAA (Indole acetic acid), kinetin and 2,4-D (2,4-dichlorophenoxy acetic acid) on Murashige and Skoog (1962) basal media. For example in a report by Prabhakaran *et al.,* (2011) on an unspecified cultivar of pigeon pea, 1 mg/l IAA in combination with a range of kinetin concentrations (0.5 -0.9 mg/l) was tested, whereas 0.6-1.0 mg/l 2, 4-D was tested alone. The highest percentage (95%) of callus formation was recorded on MS media augmented with IAA (1.0 mg/l) and Kinetin (0.9 mg/l) and 90% on MS medium with 2, 4-D (1 mg/l). Thu *et al.,* (2003) reported callus induction from surface sterilized ICPL 87 seeds and embryo axes on B5 medium containing 3% sucrose, 0.3% phytagel and various BAP concentrations (0, 0.2, 2, 5, 10 mg/l). Callus only developed on B5 medium with 10 mg/l BAP. In their experiments, no callus was recorded from embryo axes on hormone free MS medium, limited callus development was recorded on MS with 0.2 to 2 mg/l BAP, while 5 to 10 mg/l BAP gave callus that could not develop further. For mature seeds, callus only developed on B5 with 10 mg/l BAP after 20 days.

Somaclonal variation -

Somaclonal variation can be of great use in producing new crop varieties (Evans and Sharp, 1986). Depending on the culture conditions and the source of the explants, somaclonal variants of economic importance can be obtained. The origin and expression of various observed variations is very diverse. One of the more frequent variations is difference in chromosomal number e.g. aneuploidy, mixoploidy and polyploidy. Some of the causes of somaclonal variation include: lengthy culture periods, growth regulators used or nutritional stress. The result is change in the number of chromosomes or the position of the chromosomes.

Somaclonal variation for varietal improvement in pigeon pea has been exploited. Cotyledon explants were used and the progeny screened for variability. The issue culture produced different mutation events resulting in both dominant and recessive alleles. Significant variation in seed mass, plant height and damage due to *Helicoverpa armigera* were obtained when compared to the seed derived control populations. Results obtained in R1, R2 and R3 generations indicated a definite gene for white seed coat, reduced plant height, a possibility of additional genes for pest resistance and increased seed mass.

ENHANCEMENT OF PIGEON PEA GERMPLASM THROUGH WIDE CROSSES

Moose & Mumm suggested that genetic variation can be generated from segregating populations, use of unadapted exotic germplasm, transgenic events and distant interspecific crosses for widening the genetic base of commercial cultivars. In Pigeon pea, existing variability among cultivated varieties has been exploited to reach to a desirable level of productivity today. Wild species of Pigeon pea have contributed traits for high protein content, cleistogamy, dwarfing habit and cytoplasmic male sterility. Five unique cytoplasmic male sterile (CMS) systems have been derived from wild *Cajanus* species. Four wild relatives of Pigeon pea have been successfully utilized in developing cytoplasmic genetic male sterile (CGMS) lines.

Rigorous efforts have also been made to transfer resistance to *Helicoverpa armigera* from *Cajanus scarabaeoides*, *C. acutifolius* and *C. platycarpus* to the cultivated gene pool for widening the genetic base of Pigeon pea. Saxena et al. reported a partially cleistogamous line which showed less than one percent cross pollination. They purified breeding population of a cross between *C. cajan x C. lineatus* and registered three (ICPL 87018, ICPL87047 and ICPL 87154) germplasm lines with this trait. Likewise, high protein content lines ICPL 87162 and ICPL 88075 were developed from the cross of *C. cajan* x *C. scarabaeoides*. Other important traits found in wild species are root knot nematode resistance and salinity resistance. Information is also available on the transfer of pod borer resistance from *Cajanus scarabaeoides and platycarpus* species. Mallikarjuna et al. and Mallikarjuna & Moss utilized *Cajanus platycarpus* with the help of hormone aided pollinations and embryo rescue approaches to transfer *Phytophthora* blight resistance.

GENOMIC RESOURCES

DNA markers are important tools to study the geographical distribution, cultivar identification, genetic diversity and linkage analysis, gene tagging, marker assisted selection and association mapping. Pigeon pea genomic initiative has focussed mainly on the development of robust set of molecular markers including microsatellites, single nucleotide polymorphisms (SNPs) and diversity array technology markers. Dubey et al. developed large number of microsatellite markers from BAC-end sequences and microsatellite enriched libraries. They have generated about 496,705 sequence reads and 10,000 Sanger ESTs from *Fusarium* wilt (FW) and sterility mosaic (SM) samples that resulted into 4,557 unigenes. Similarly, Ganesha et al. developed the intra-specific genetic maps and identified QTLs for SMD resistance. Yang et al. used DArT analysis for cultivar identification and differentiation between regions and place of origins. They observed morphological variation in cultivated germplasm was much higher than that at molecular level and the wild related species revealed substantial molecular diversity than that observed at morphological level. Varshney et al. have published the draft sequence of Pigeon pea and predicted 48,680 genes and their potential role in unravelling drought tolerance, domestication of Pigeon pea and the evolution of its ancestors.

MATURATION AND GERMINATION IN PIGEONPEA (*CAJANUS CAJAN)*

Pigeon pea (Cajanus cajan L. Millsp) is one of the most popular legume grains in the world, especially in the Indian subcontinent. Due to its multiple uses, pigeon pea is widely used in intercropping systems in semi-arid regions. It provides the main source of protein for many of the poorest populations and plays an important role in
reducing malnutrition for millions people around the world. Pulses can furnish an eminent source of dietary protein constituents for human consumption as a big benefit in a balanced energy and protein diet for those who live in developing countries, especially when intake from animal or fish sources is limited or insufficient, hi Egypt, pigeon pea is classified as a non-traditional crop, though It has a multiple uses mainly under the newly reclaimed sandy soil, rather than the old cultivated soils, Attempts to obtain stress-resistant genotypes of pigeon pea species by conventional breeding methods have not been successful because of limited genetic variation and sexual incompatibility with wild relatives, The availability of an in vitro regeneration protocol is a pre-requisite for the application of most

biotechnological techniques, such as production of synthetic seed, transgenic plants and somaclones. Genetic engineering approaches to introduce genes coding for insecticidal proteins into pigeon pea may prove useful in obtaining pest-resistant genotypes, A prerequisite for the genetic transformation of a crop system is the availability of a good protocol for in vitro plant regeneration. In most cases the somatic embryos or the embryogenic cultures can be cryopreserved which makes it possible to establish gene banks, Furthermore, in vitro somatic embryogenesis is an important prerequisite for the use of many biotechnological tools for genetic improvement, In this regard, somatic embryos play a key role in current genetic transformation methods. The development of a somatic embryogenesis technique could have a great impact on pigeon pea improvement programs.

Establishment of cultures yielding high frequency somatic embryogenesis would be useful for gene expression studies involving genetic transformation since a steady quantity of target tissue can be produced. Cell differentiation into somatic embryos is governed by environmental or cultural stimuli. Somatic embryogenesis offers an alternative and efficient means for plant multiplication. The present study describes a reproducible protocol for plant regeneration via somatic embryogenesis, through hypocotyls explant in two important genotypes of pigeon pea.

REGENERATION PROTOCOL FOR PIGEONPEA

Pigeonpea (*Cajanus Cajan* [L.] Millispaugh*)* is an important grain legume of family Fabaceae. It is an out-crossed, diploid (2n=2x=22) crop with genome size of 800 Mbp. Pigeonpea is mainly cultivated in tropical and subtropical regions of the world. Globally pigeonpea is cultivated on 4.6 mh with annual production of 3.25 mt. India accounts for 78% of the global output with current production of 2.9 mt from 4.4 mh. In India, pigeonpea is mainly grown in states of Madhya Pradesh, Uttar Pradesh, Rajasthan, Karnataka and Andhra Pradesh. Pigeonpea is rich in protein (20-22%) particularly sulphur containing amino acids, namely methionine and cysteine (Singh *et al*., 1990). Besides, seeds also contain about 57.3-58.7% carbohydrates, 1.2-8.1% crude fibres and 0.6-3.8% lipids. The pigeonpea varieties are broadly categorized into three classes based on duration of maturity *viz*. *early duration* (140-150 days), *medium duration* (160-200 days) and *late duration* varieties (more than 200 days). It serves as a host for silkworm (Madagascar) and the lac insect. Stems and branches, especially those of medium- and long-duration cultivars, are used for basketry, thatching, fencing and as fuel. In Nigeria the stems serve as stakes for yam. Pigeonpea finds wide application in traditional medicine. Diarrhoea, gonorrhoea, measles, burns, eye infections, earache, sore throat, sore gums, toothache, anaemia, intestinal worms, dizziness and epilepsy are treated with leaf preparations, root preparations are taken to treat cough, stomach problems and syphilis, stem ash for wounds, and stalks and roots are chewed against toothache. Powdered seeds serve as a poultice on swellings. In Madagascar the leaves are used to clean teeth. In post green revolution period, the per capita availability of pigeonpea has declined in the country mainly due to widening demand–supply gap caused by mismatch in population and production growths. In spite of being the largest producer of pigeonpea in the world, the average productivity in the country is mere 745 kg/ha. The major constraints that limit farmers from achieving potential yield of pigeonpea includes non-availability of quality seeds of improved varieties in adequate quantity, poor crop management, and biotic and abiotic stresses prevalent in the pigeonpea growing areas besides socio–economic factors.

The most important fungal diseases of pigeonpea are leaf spot (*Mycovellosiella cajani*,), *Fusarium* wilt (*Fusarium udum*), powdery mildew (*Leveillula taurica*), root-knot nematodes *(Meloidogyne* spp.) and reniform nematodes (*Rotylenchus* spp.). Insect pests like pod-sucking bugs (*Clavigralla* spp.), pod borers (*Helicoverpa armigera* and *Maruca vitrata*) and pod fly (*Melanagromyza chalcosoma*). Among abiotic stresses, water logging during vegetative stage, cold sensitivity during flowering stage, terminal drought during grain filling stage and salinity/alkalinity throughout the crop period inflict major yield losses and instability in production. All these stresses make the crop less productive with unstable performance. Besides biotic and abiotic stresses, low initial crop growth and low harvest index also limit its yield production. So, due to various stresses the production of this valuable crop is constrained therefore attempts for *in vitro* regeneration are done. *In vitro* regeneration protocol is affected by type of explants, genotype, hormonal combination and temperature and light conditions. This *in vitro* regeneration protocol also helps for development of transgenic protocol and production of transgenic plants. Thus, the present review provides an overview of current knowledge concerning *in vitro* regeneration protocol in pigeonpea.

Pigeonpea Regeneration- Pigeonpea regeneration is affected by pathway of regeneration, genotypes, culture conditions and combination of growth regulator selected for developing the protocol.

Pathways of Regeneration- Various protocols for plant tissue culture have been developed during the past five decades and any plant species can now be regenerated *in vitro* through several pathways. Various pathways have been put forward to depict the regeneration processes such as organogenesis and somatic embryogenesis. Cells, tissues and

organs from numerous plant species can be successfully cultured aseptically to regenerate whole plants.

CONCLUSION

After, reviewing the available literature on pigeonpea regeneration thoroughly it was observed that cotyledonary node explant proved to be most suitable for *in vitro* regeneration as it showed high regeneration frequency. MS media was the most suitable medium for tissue culture based studies on several plant species.

The novelty of the cited research shows that combination of cytokinin i.e. zeatin and kinetin responded vigorously to shoot induction for cotyledornary node, embryonic axis and Scutellum explants, which was earlier reported incredibly time consuming through a variety of auxin and cytokinin combination. The shoot proliferation with silver nitrate as additives expose at superior velocity. The above three mention explants work out on single hormonal combination for regeneration and no reports were found with such singlehandedly combination . Root induction was produced within little time period of which is prerequisite for hardening. This was the first report to employ silver nitrate along with cytokinin combination which facilitates regeneration with elevated velocity within stipulated time.

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