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In vitro REGENERATION OF PIGEON PEA USING LEAF EXPLANTS

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ABSTRACT

Pigeon pea (*Cajanus cajan* (L.) Millsp.) is a drought tolerant pulse legume, mainly grown for grain in the semi-arid tropics, particularly in Africa. Pigeon pea production in countries like Kenya is faced with a number of challenges, particularly lack of high quality seeds. The objective of this study was to develop an *in vitro* regeneration system for pigeon pea varieties grown in Kenya, that is amenable to genetic transformation. *In vitro* regeneration of pigeon pea varieties, KAT 60/8 and ICEAP 00557, commonly grown in Kenya was achieved using leaf explants from *in vitro* grown seedlings, through callus initiation, followed by shoot and root induction. For callus initiation, MS media supplemented with 0.5-4 mg l^{-1} 2, 4-D and TDZ separately were tested, and IBA at 0.1, 0.5 and 1 mg l^{-1} was tested for rooting of shoots. Embryogenic calli was obtained on MS containing 2, 4-D; whereas TDZ induced non-embryogenic callus alone or with shoots directly on explants. Indirect shoot regeneration frequency of 6.7 % was achieved using 1 mg l^{-1} 2, 4-D-induced embryogenic callus obtained using KAT 60/8 explants. Whereas direct shoot regeneration frequencies of 20 and 16.7% were achieved using ICEAP 00557 and KAT 60/8 explants, using 0.5 mg l^{-1} and 2 mg l^{-1} TDZ, respectively. Optimum rooting was achieved using 0.5 mg l^{-1} IBA; and up to 92% rooted shoots were successfully established in soil after acclimatisation. Genotype and hormone concentrations had a significant ($P<0.05$) influence on callus, shoot and root induction. The protocol developed can be optimised for mass production and genetic transformation of KAT 60/8 variety.

Key Words: 2, 4- D, embryogenic callus, shoot regeneration

RÉSUMÉ

Le pois d'angole (*Cajanus cajan* (L.) Millsp.) est une espèce de légumineuse tolérant la sécheresse, cultivée essentiellement dans les zones tropicales semi-arides de l'Afrique. La production du pois d'angole dans des pays comme le Kenya est sujette à des nombreuses difficultés, notamment, le manque de semences de qualité. L'objectif de cette étude était de développer une procédure de régénération *in vitro* des variétés de pois d'angole cultivées au Kenya, en vue d'opérer des transformations génétiques. La reproduction *In vitro* de KAT 60/8 et ICEAP 00557, variétés fréquemment cultivées au Kenya a été réalisée en se servant d'explants de feuilles provenant des graines germées *in vitro*, au travers de la procédure d'initiation de callosités, suivie de followed d'induction de la régénération méristématique et racinaire. Pour l'initiation des callosités, le media MS enrichi avec 0,5-4 mg l^{-1} de 2, 4-D et TDZ ont été testés séparément, et IBA à 0,1, 0,5 et 1 mg l^{-1} avaient été testé pour la production racinaire. Des callosités embryogéniques ont été obtenues sur media MS contenant 2, 4- D; tandis que TDZ ne permettait que de régénérer des callosités non embryogéniques ou avec des pousses directement sur explants. La fréquence de régénération indirecte des pousses réalisée était de 6,7 % avec 1 mg l-1 de 2, 4-D sur des callosités embryogéniques prélevées sur explants de KAT 60/8. Tandis que des fréquences de régénération directe de pousses étaient respectivement de 20 et 16.7% avec des explants de ICEAP 00557 et KAT 60/8, sur 0.5 mg l^{-1} et 2 mg l^{-1} de TDZ. La régénération racinaire était optimale sur 0.5 mg l^{-1} IBA; et jusqu'à 92% des pousses se sont insérés dans le sol

avec succès après l'acclimatation. La concentration hormonale et le génotype avaient des influences significatives (P<*0.05*) sur l'induction de pousses et de racines au niveau des callosités. Le protocole développé peut être adapté à la production de masse et à des transformations géniques de la variété KAT 60/8.

Mots Clés: 2, 4- D, callosité embryogénique, régénération racinaire

INTRODUCTION

Pigeon pea (*Cajanus cajan* (L) Millsp.) is a drought tolerant pulse legume mainly grown for grain in the semi-arid tropics (Nene and Sheila, 1990; Pambo, 2014). It is an important food security crop for local consumption and export for several African countries, particularly Kenya, Malawi, Mozambique, Tanzania and Uganda (Kaoneka *et al*., 2016).

Pigeon pea production in Kenya is faced with a number of challenges, particularly lack of high quality seeds. Production constrains are further exacerbated by susceptibility of the crop to pests like pod borers, and diseases like *Fusarium* wilt. The available option for controlling insect pests and diseases is by use of chemicals, which are expensive and unaffordable for most small scale resource limited farmers (Mergeai *et al*., 2001; Sharma *et al*., 2010). The main pigeon pea insect pest in Kenya is the pod borer (*Helicoverpa armigera*), which causes extensive damage to the crop (Lateef and Reed, 1983; Parde *et al.,* 2012).

Pod sucking bugs and thrips can cause up to 78 and 47% yield loss, respectively (Rotimi and Iloba, 2008; Dialoke *et al*., 2010). There have been attempts to develop genotypes that are resistant to *H. armigera* through conventional breeding in Kenya; with limited success due to narrow genetic diversity and sexual incompatibility associated with cultivated varieties and their wild relatives (Nene and Sheila, 1990; Varshney *et al.,* 2010).

Grain legumes are generally considered to be recalcitrant to *in vitro* regeneration and transformation (Veltcheva *et al*., 2005; Hnatuszko-Konka *et al*., 2014); though some successes have been reported. Through *in vitro* regeneration of pigeon pea, somaclonal variants that are resistant to *Fusarium* wilt and *H. armigera* can be obtained (Chintapalli *et al.,* 1997). A reproducible *in vitro* regeneration protocol is also a pre-requisite for genetic transformation of many crop species. It facilitates gene transfer, selection and regeneration of transformants to be achieved. Therefore, new genes for economically important traits, such as resistance to diseases and pests, can be introduced (Sreenivasu *et al.,* 1998; Krishna *et al*., 2011a. Therefore the objective of this study was to develop an *in vitro* regeneration system for pigeon pea varieties that is amenable to genetic transformation.

MATERIALS AND METHODS

Plant material. Mature seeds of commercial pigeon pea (*Cajanus cajan),* varieties KAT 60/8 and ICEAP 00557, were obtained from the Kenya Agricultural and Livestock Research Organisation (KALRO), Katumani, in Kenya. Seeds (50 seeds per wash) were washed under running tap water, for 5 minutes; and then rinsed with sterile distilled water. Three drops of teepol® (Sodium Dodecylbenzene Sulphonate) and three drops of sodium hypochlorite (10 % w/v) were added and swirled for 10 minutes before decanting the water. The seeds were then rinsed in three sequences of sterile distilled water, before shaking in mercuric chloride (0.1%w/v) for five minutes. They were then rinsed in four series of sterile distilled water, before germinating them aseptically on plant growth regulator-free MS medium (PGRs-free MS). The seeds were germinated and grown for 10 days in a growth chamber, with a photoperiod of 16 hours, provided by Phillips® cool daylight fluorescent tubes (ca. 60 $\mu M\ m^2\ s^{-1}$), and a temperature of 28± 2 °C. The 10-day old seedlings served as a source of leaf explants. The first two leaves of every seedling were harvested and their margins carefully trimmed before slicing the leaf blades into segments of approximately 5 mm^2 using a sterile scalpel blade, which were then immediately inoculated with the abaxial side in contact with the respective callus induction media.

Callus induction and plant regeneration. The basic media used for callus induction consisted

of Murashige and Skoog (1962) basal salts and vitamins (sourced from Duchefa Biochemie B.V., Netherlands), supplemented with 3 % (w/v) sucrose, 0.8% (w/v) agar (Thomas Baker, India), glutamine (200 mg l^{-1}), casein hydrosylate (500 mg l^{-1}) and 10 mg l^{-1} silver nitrate. The MS media containing thidiazuron (TDZ) at 0.5, 1.0, 2.0 and 4.0 mg l^{-1}, and 2, 4-D at 0.5, 1.0, 2.0 and 4.0 mg l^{-1} were tested separately for induction of embryogenic callus. The pH of the media was adjusted to 5.8, using 1 M HCl or 1 M NaOH, prior to autoclaving for 20 minutes at 120 °C at 15 pounds per square inch (psi), and 50 ml of media dispensed into sterile 500 ml glass jars inside laminar flow cabinet.

For shoot induction, the callus formed was excised from the leaf explants and transferred onto MS medium containing BAP at 0, 0.1 and 0.5 mg l^{-1}, and then cultured for 40 days. The shoots formed were excised and transferred to MS medium containing 0.4 mg l^{-1} gibberellic acid or 0.1 mg l^{-1} BAP for elongation. The influence of MS medium containing different concentrations of IBA (0, 0.1, 0.5, 1.0 mg l^{-1}) on root induction of the elongated shoots, was tested. Plantlets with sufficient roots were carefully removed from the culture vessels, roots washed with sterile dH_2O and then transplanted into pots containing sterile soil mixed with vermiculite (1:1) for 21 days. The pots were covered with perforated polythene bags for 10 days and watered with ¼ MS twice a day. The polythene bags were removed and the plants allowed to establish under greenhouse conditions.

Culture conditions and data analysis. Embryogenic callus, shoot and root induction cultures were incubated in a growth cabinet with similar temperature and photoperiod conditions as described for seed germination. All experiments were conducted in a completely randomised design. For embryogenic callus and shoot induction, five independent repeated experiments were conducted, and for each experiment 30 explants were inoculated per treatment.

Data on embryogenic callus and shoot induction frequencies, and number of roots per shoot were collected. Callus and shoot induction frequencies were determined based on the number of leaf explants with embryonic callus and callus with shoot regenerations, respectively, over total explants inoculated per treatment. During data analysis each repeat experiment was treated as a replicate. Data for callus and root induction frequencies were arcsine transformed prior to ANOVA using GenStat ver.12 software (completely randomised design option). Fisher's Least Significant Difference (LSD) test was used ($P<0.05$) to compare the means.

RESULTS

Callus induction efficiency. An initial seed germination of more than 95% was achieved with surface sterilised ICEAP 00557 and KAT 60/8 seeds at 4 days after inoculation, on PGRs-free MS medium, although up to 100% germination was recorded after 7 days of culture (Table 1).

TABLE 1. Germination of surface sterilised seeds of ICEAP 00557 and KAT 60/8 varieties on plant growth regulator free-free MS media

Variety	Experiment	No. of seedsInoculated	Germination (%)		
			Day 3	Day 4	Day 7
ICEAP 00557	1	50	66	98	100
	2	50	65	100	100
	3	50	76	96	100
KAT 60/8	1	50	72	96	100
	2	50	70	95	98
	3	50	74	95	100

There were no bacterial or fungal contaminants observed after 10 days of culture; hence leaf explants obtained from the seedlings for subsequent experimentation were free from contamination. During callus induction, 13.3 and 6.7 % of the leaf explants, for KAT 60/8 and ICEAP 00557, respectively, inoculated on MS basal medium enlarged and developed white compact non-embryogenic callus (Table 2). However 86.7 and 93.3% of KAT 60/8 and ICEAP 00557 leaf explants, respectively, enlarged but did not form callus. On the other hand, KAT 60/8 and ICEAP 00557 leaf explants inoculated on medium with 0.5, 1, 2 and 4 mg l^{-1} 2, 4-D concentrations, produced pale-yellow embryogenic calli (Fig. 1A). On media containing TDZ alone, KAT 60/8 leaf explants generally produced white compact non-embryogenic callus that appeared mainly on the cut margins, after 20 days of culturing (Fig. 1B). In addition, shoot buds were induced directly on the explant surface, mainly localised around the midrib and the petiolar areas on medium containing 0.5 mg l^{-1} TDZ. ICEAP 00557 explants also formed compact non-embryogenic callus on the cut margins on medium containing TDZ and shoot buds were also evident on the explant surface.

Irrespective of callus type induced (non- or embryogenic callus), the concentration of PGRs and pigeon pea genotype tested had a significant ($P<0.05$) influence on callus induction. Furthermore, interactions between PGRs type and concentration and, between variety, PGRs type and concentration also had a significant ($P<0.05$) influence on callus induction. Generally, increasing the concentration of 2,4-D or TDZ resulted in increased callus induction efficiency (Table 2), except for ICEAP 00557 variety, where an inverse response was observed on treatments with >1 mg l^{-1} 2,4-D. For KAT 60/8 variety, irrespective of the PGR type and concentrations tested, callus induction frequencies of up to 90% were obtained. However, for ICEAP 00557, only explants inoculated on medium containing 2 and 4 mg l^{-1} TDZ recorded a callus induction frequency of 90 %, unlike for 2,4-D, where the highest callus induction was obtained with 1 mg l^{-1} (Table 2). Although the highest induction efficiency of 80.36% was obtained with ICEAP 00557 for leaf explants cultured on 1 mg l^{-1} 2,4-D, this efficiency

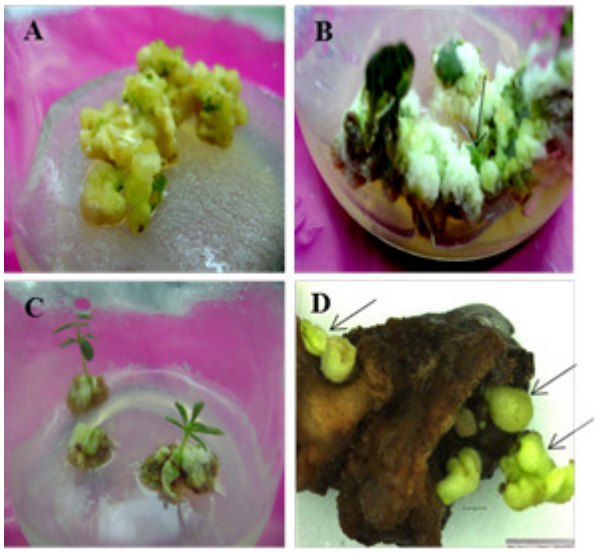

Figure 1. Callus induction and shoot regeneration of pigeon pea using leaf disc explants obtained from 7 day old *in vitro* germinated seedlings. (A) Pale-yellow embryogenic callus induced using 1 mg l^{-1} 2,4- D; (B) Forty two day old non-embryogenic callus and emerging shoots on callus after leaf explants were cultured on MS media containing 0.5 mg l^{-1} TDZ for 42 days; (C) Elongation of callus-regenerated shoots after 28 days of culture on MS medium containing 0.1 mg l^{-1} BAP; (D) Induction of direct somatic embryos (indicated by arrows) on PGR-free and 0.1 mg l^{-1}BAP medium after 28 days of culture using leaf explants initially cultured on MS containing 2.0 mg l^{-1} TDZ for 75 days. (Bar = 1 mm).

TABLE 2. Callus induction efficiency using leaf explants obtained from *in vitro* grown 10 day-old pigeon pea KAT 60/8 and ICEAP 00557 seedlings on MS medium supplemented with 2,4-D and TDZ

Pigeon pea variety	PGR type	PGR (mg l^{-1})	Callus induction (%)
ICEAP 00557	TDZ	0	9.64
		0.5	62.07
		1.0	71.36
		2.0	90
		4.0	90
	2,4-D	0	16.69
		0.5	69.13
		1.0	80.36
		2.0	63.90
		4.0	51.13
KAT 60/8	TDZ	0	11.87
		0.5	76.18
		1.0	90
		2.0	90
		4.0	90
	2,4-D	0	18.92
		0.5	80.36
		1.0	90
		2.0	90
		4.0	90
CV (%)			19.1
LSD (0.05)			16.01

Values are means of 5 repeat experiements (30 explant per treatment) in their original scale. Data were transformed using arcsin prior to ANOVA. PGR = Plant growth regulator

was not significantly ($P<0.05$) different from treatments with scores of 90 % (Table 2).

Shoot regeneration. A shoot regeneration frequency of 6.7% was achieved when KAT 60/8 embryogenic callus induced using 1.0 mg l^{-1} 2, 4-D were sub-cultured on regeneration media containing 0.5 mg l^{-1} BAP. Although embryogenic callus were also successfully induced using 2, 4-D at 0.5, 2.0 and 4.0 mg l^{-1}, they failed to produce shoots when sub-cultured onto shoot regeneration media. The regenerated shoots were successfully elongated on 0.1 mg l^{-1} BAP for 30 days (Fig. 1C). On the other hand, a shoot regeneration frequency of 10% was achieved when KAT 60/8 leaf explants, with non-embryogenic calli induced using 0.5 mg l^{-1} TDZ, were transferred onto PGR-free medium. In addition, 40% of the explants with calli produced numerous non-harvestable shoots and the remainder (50 %) became necrotic, between the 21 and 35 days after transfer to PGR-free medium. The non-embryogenic callus induced using 1.0, 2.0 and 4.0 mg l^{-1} TDZ, turned brown without producing shoots, after 21 days of culture on PGR-free medium.

The KAT 60/8 leaf explants cultured on 0.5 mg l^{-1} TDZ for 20 days, formed callus on the trimmed margins. However, when these explants were transferred on to shoot regeneration medium (0.1 mg l^{-1} BAP) for 40 days, numerous shoot buds emerged directly from the leaf surface. In contrast, no shoot buds were obtained from leaf explants initially cultured on 1.0 mg l^{-1} TDZ, but only led to development of white compact callus that turned dark brown after 21 days. For those KAT 60/8 leaf explants that were initially cultured on 2.0 mg l^{-1} TDZ, shoots emerged from the trimmed surfaces of the leaf explants. A shoot regeneration frequency of 16.7 % was obtained when KAT 60/8 leaf explants comprising of compact non embryogenic callus induced using 2.0 mg l^{-1} TDZ were transferred on to regeneration medium containing 0.1 mg l^{-1} BAP. In contrast no shoot were obtained when KAT 60/8 leaf explants, comprising of 4.0 mg l^{-1} TDZ-induced compact non embryogenic callus were transferred to 0.1 mg l^{-1} BAP.

Unlike KAT 60/8, no shoots were induced when ICEAP 00557 callus produced using 0.5,1.0, 2.0 and 4.0 mg l^{-1} 2, 4-D, were transferred onto shoot regeneration media consisting of 0.1 or 0.5 mg l^{-1} BAP. Similar to KAT 60/8, when ICEAP 00557 non-embryogenic callus induced on MS medium containing 1.0, 2.0 and 4.0 mg l^{-1} TDZ were transferred to PGR-free medium, they failed to produce shoots. However, a 20% shoot regeneration frequency was achieved when ICEAP 00557 leaf explants, comprising of non-embryogenic callus induced using 0.5 mg l^{-1} TDZ, were transferred to medium containing 0.1 mg l^{-1} BAP. The shoots were formed directly on the leaf explants. Furthermore, leaf explants comprising of callus induced on medium containing 2.0 and 4.0 mg l^{-1} TDZ had a shoot regeneration frequency of 6.0% after transfer to medium containing 0.1 mg l^{-1} BAP. However, no shoots

appeared when leaf explants, comprising of callus induced on medium containing 1.0 mg l^{-1} TDZ, were transferred onto the shoot induction medium containing 0.1 mg l^{-1} BAP. On the other hand, when ICEAP 00557 and KAT 60/8 leaf explants were cultured for 75 days, those on MS containing 2.0 mg l^{-1} TDZ produced clusters of somatic embryos at 28 days after transferred to PGRs-free MS and medium containing 0.1 mg l^{-1} BAP. A microscopic view of the somatic embryos displayed different developmental stages, ranging from globular to torpedo-shaped (Fig. 1D). However, transfer of the somatic embryos to ½ MS and MS with 0.5 mg l^{-1} BAP for 56 days only, increased their sizes but no germination was attained.

Root induction. KAT 60/8 and ICEAP 00557 shoots displayed slow and scanty root development, when inoculated in PGR-free MS medium (Fig. 2D). However, the roots had lateral branches which appeared normal and did not have callus on the surface. On the medium with 0.1 mg l^{-1} IBA, shoots for both varieties produced more roots with similar architecture, compared with those in PGR-free treatment, although some callused (Fig. 2A). Profuse root induction was observed on shoots cultured on to MS media containing 0.5 mg l^{-1} IBA, for both KAT 60/8 and ICEAP 00557. The roots were also laterally branched, and thicker than those induced on PGR-free medium due to callusing (Fig. 2B). They also had more roots per shoot than those on MS

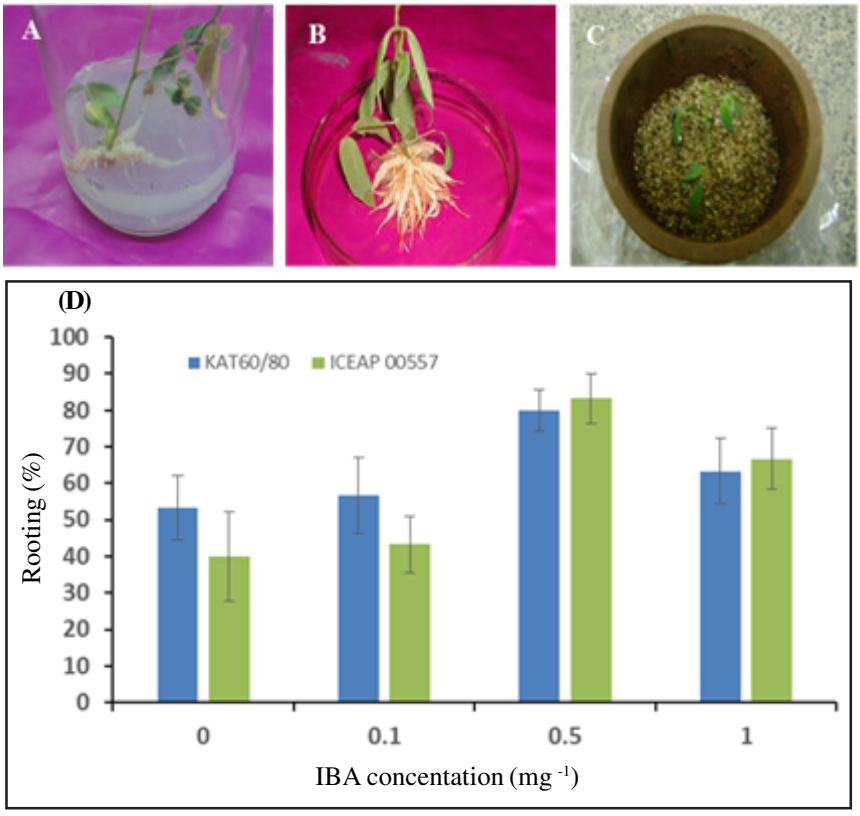

Figure 2. Root induction and hardening of regenerated pigeon pea plantlets. (A) Induction of roots from *in vitro* grown micro shoots; (B) Profuse root induction and callusing on MS augmented with 0.5 mg l^{-1} IBA at 28 days after inoculation of shoots; (C) Hardening of rooted pigeon pea shoots in pots containing sterile vermiculite at 14 days after transfer; (D) KAT 60/8 and ICEAP 00557 root induction frequencies on MS medium containing different concentrations of IBA. Data was arcsin transformed prior to ANOVA. Bars on the graph represent SEM and graphs with different letters are significantly ($P<0.05$).

TABLE 3. Survival of regenerated ICEAP 00557 and KAT 60/8 plantlets transplanted on soil-vermiculite mixture (1:1) and grown under greenhouse conditions

Variety	Plantlets transplanted	Plantlets surviving (%)			
		Day 7	Day 14	Day 21	Day 30
ICEAP 00557	24	96	96	96	92
KAT 60/8	20	100	90	85	80

with 0.1 mg l^{-1} IBA (Fig. 2B). Similarly, in the root induction medium containing 1.0 mg l^{-1} IBA, both KAT 60/8 and ICEAP 00557 shoots had laterally branched callused roots, with the same architecture as those observed on medium containing 0.5 mg l^{-1} IBA. Plantlets with non- and callused roots, established well in vermiculite: soil potting mixture after 30 days of weaning (Fig. 2C). A survival rate of 92 and 80% was achieved on ICEAP 00557 and KAT 60/8, respectively; after weaning the regenerated plants under greenhouse conditions (Table 3).

Generally, increase in the concentration of IBA tested up to 0.5 mg l^{-1}, resulted in higher root induction frequencies (Fig. 2D). Root induction was significantly ($P<0.05$) influenced by the concentration of IBA only, while Fisher's LSD ($P<0.05$) test revealed that root induction efficiency at 0.5 mg l^{-1} IBA was significantly ($P<0.05$) different from other IBA concentrations tested (Fig. 2D).

DISCUSSION

Callus induction efficiency. The callus induction frequencies of 13.3 and 6.7%, achieved with KAT 60/8 and ICEAP 00557 varieties, respectively, on PGRs-free medium (Table 2), suggest presence of endogenous PGRs at a level high enough to induce callus in the absence of exogenous PGRs. However, the number of explants forming callus were quite few, as demonstrated by the low induction frequencies achieved, irrespective of the pigeon pea genotype. The presence of auxins in a growing leaf has been reported widely (Ljung *et al.,* 2001; Heisler *et al.,* 2005), and generally, leaf development comprises of two events namely, meristematic and vascular differentiation (Scarpella *et al.,* 2010). These two activities have been shown to correlate with production of auxins in a leaf, particularly during xylem differentiation, and hence more auxins tend to be present in the vein rather than lamina regions (Ljung *et al*., 2001). The ICEAP 00557 and KAT 60/8 explants were obtained from leaves of 10-day old seedlings, these leaves were still undergoing differentiation. Therefore, the low callus induction frequencies obtained on the PGR-free medium, clearly suggest variation in the apportioning of the endogenous PGRs in the 10-day old pigeon pea leaves. Since leaf explants were obtained randomly across young developing leaf blade, explants with vein tissues had more auxin levels, most probably IAA (Chudasama and Thaker, 2007; Mano and Nemoto, 2012) that was responsible for initiating callus in the absence of exogenous PGRs.

Irrespective of the PGR tested, relatively lower callus induction frequencies were achieved when explants from ICEAP 00557 seedlings were used (Table 2). This was particularly more pronounced on media containing auxin 2,4-D. High plasticity for cell differentiation is one the fundamental characteristic of plant cells, which allows plants to develop disorganised cell masses like callus (Ikeuchi *et al*., 2013). However, callus induction and its regeneration potential is influenced by a multiple of factors, which include PGR-type and concentration, genotype, explant, carbohydrate sources and basal salts of culture medium (Aboshama, 2011). Therefore, variation in callus induction frequencies observed with ICEAP 00557 and KAT 60/8 leaf explants were mainly attributed to the effect of genotype and PGR type concentrations.

A similar response has been reported on Egyptian pigeon pea varieties, ICPL 87-118 and ICPL 151, using mature cotyledon explants (Aboshama, 2011). For callus induction, an auxin is generally required (Thorpe *et al.,* 2008) and all the 2, 4-D concentrations tested induced

embryogenic callus; however, only those induced using 1.0 mg^{-1} 2, 4-D produced a low shoot regeneration frequency of 6.67 % only for variety KAT 60/8.

The induction of embryogenic callus is in line with many reports indicating that 2, 4-D alone or in combination with lower level of cytokinins are effective in inducing embryogenic callus for many plant species (Brown *et al*., 1995). However, induction of embryogenic callus did not automatically lead to regeneration of shoots, using lower concentrations of BAP. These results suggest that regeneration of shoots from the embryogenic callus is genotype dependent. There is, however, need to screen other types and concentration of cytokinins on inducing regeneration of shoots from the 2, 4-D induced embryogenic callus.

Similar to induction of callus on PGRs-free medium, the appearance of shoot buds near the veins for KAT 60/8 and ICEAP 00557 leaf explants, inoculated on media containing TDZ, was attributed to the interaction of the exogenous cytokinin (TDZ), with low levels of endogenous auxins, in parts of explants with vein tissue. TDZ, in combination with low auxins, generally induce shoot formation on explants (Srivastava, 2013). TDZ is categorised as a cytokinin because of its induction of cytokinins-like responses, such as initiation of shoot regeneration (Guo *et al*., 2013), a response that is evident in our study. However, the shoot regeneration frequencies recorded were relatively low may be due to the effect of genotypes. On the other hand, induction of somatic embryos on leaf explants of both KAT 60/8 and ICEAP 00557 varieties on media containing 2.0 mg l^{-1} TDZ is a response usually initiated by relatively higher concentrations of suitable auxin or auxin to cytokinin combinations.

The morpho-regulatory potential of TDZ as a cytokinin, and its efficiency in inducing plant cell dedifferentiation, have led to its use in *in vitro* regeneration of various plant species. However, the results obtained with leaf explants of KAT 60/8 and ICEAP 00557 (Table 2) clearly demonstrate that TDZ has auxin-like activity that initiated the production of non-embryogenic callus and somatic embryos (Fig. 1D) These results confirmed report by Aboshama (2011), indicating that TDZ has both cytokinin and auxin-like activity. These results confirmed a report by Aboshama (2011) indicating that TDZ has both cytokinin and auxin-like activity, thus, promoting cell division and differentiation, in addition to inducing embryogenic competence in callus tissue. In addition to callus initiation, lower concentrations of TDZ (0.5 mg l^{-1}) also induced direct shoot organogenesis. This is also in line with reports by Aboshama (2011), in which prolonged culturing of cotyledonary explants of pigeon pea variety ICPL 151 in medium containing TDZ produced the same response.

The somatic embryos obtained in this study were similar to those described by Aboshama (2011) from pigeon pea varieties ICPL 151 and ICPL 87-118, and Sreenivasu *et al.* (1998) using Pusa cultivars. However, no germination was achieved on the different media tested; could be attributed to lack of maturation treatment with abscisic acid, which has been used in other studies to increase the number of normal germinated somatic embryos significantly (Aboshama 2011; Mohan and Krushnamurthy, 2002). Successful regeneration of pigeon pea, a recalcitrant legume, through somatic embryogenesis, was described for the first time in 1998 from cotyledon and leaf explants of Pusa 606 cultivar (Sreenivasu *et al.,* 1998), following an earlier attempt by Nalini *et al*., (1996) who reported globular embryos that failed to produce plantlets from callus induced on immature leaflets of ICPL 87.

Root induction in many dicots is a response widely known to be triggered by exogenous application of different auxins. However, root induction for KAT 60/8 and ICEAP 00557 shoots was also obtained on PGRs-free MS medium. This response can be attributed to presence of endogenous auxins, since they are naturally produced in stems, buds and root tips (Hochholdinger and Zimmermann, 2008). The presence of endogenous auxins and their directional distribution, usually helps to synchronise proper development of roots (Overvoorde *et al*., 2010). Therefore, shoots can form roots in the absence of exogenously applied auxins, though their induction frequency is genotype dependent.

Although there was induction of roots on PGRs-free medium, there was a delay of 2 to 5

days, compared with media with PGRs. Roots developed in MS medium, containing 0.1 mg l^{-1} IBA, appeared normal like those of PGRs-free MS medium (Fig. 2D), but developed faster (3 to 4 days less) than those in PGRs-free MS medium. This response suggests presence of a positive interaction between the endogenous and exogenously applied auxin (IBA), which enhanced root emergence. Nonetheless, there are reports where PGRs-free MS liquid medium was optimal for root induction for *in vitro* regenerated shoots of Hyderabad C pigeon pea cultivar (Geetha *et al*., 1998). Supplementing MS medium with 0.5 mg l^{-1} IBA was optimal in the induction of roots for the *in vitro* regenerated KAT 60/8 and ICEAP 00557 shoots pigeon. Interestingly there are different reports indicating that IBA at 0.5 mg l^{-1} was also optimal for other pigeon pea genotypes, namely JKR105, VBN1, VBN2 and SA1 (Franklin *et al.,* 2000; Krishna *et al.*, 2011b). There are also contradicting reports indicating that IBA at 0.2 mg l^{-1} IBA and 1.0 mg l^{-1} were optimal for root induction in pigeon pea cultivars Hyderabad C and LGG-29, respectively (Geetha *et al*., 1998; Prasad *et al*., 2011).

CONCLUSION

In this study, an *in vitro* regeneration protocol for farmer preferred pigeon pea cultivar KAT 60/8 has been developed using leaf explants obtained from *in vitro* raised 10-day old seedlings. This protocol can be optimised for genetic transformation of pigeon pea KAT 60/8 for insect resistance, a variety that is highly affected by the pod sucking bugs leading to major yield losses

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REFERENCES

Aboshama, H.M.S. 2011. Somatic embryogenesis proliferation, maturation and germination in *Cajanus cajan*. *World Journal of Agricultural Sciences* 7(1): 86-96.

Brown, D.C., Finstad, K.I. and Watson, E.M. 1995. Somatic embryogenesis in herbaceous dicots. *In vitro* embryogenesis in plants. pp. 345-415. Springer Netherlands.

Chintapalli, P.L., Moss, J.P., Sharma, K.K. and Bhalla, J.K. 1997. *In vitro* culture provides additional variation for pigeon pea. *In Vitro Cellular and Developmental Biology-Plant* 33(1): 30-37.

Chudasama, R.S. and Thaker, V. 2007. Free and conjugated IAA and PAA in developing seeds of two varieties of pigeon pea (*Cajanus cajan*). *General and Applied Plant physiology* 33 (1-2): 41-57.

Dialoke, S.A., Agu, C.M., Ojiako, F.O., Onweremadu, E., Onyishi, G.O., Ozor, N., Echezona, B. C., Ofor, M.O., Ibeawuchi, I.I., Chigbundu, I.N., Ngwuta, A. A. and Ug woke, F.O. 2010. Survey of insect pests on pigeon pea in Nigeria. *Journal of SAT Agricultural Research* 8:1-8.

Franklin, G., Jeyachandran, R. and Ignicimuthu, S. 2000. Factors affecting regeneration of pigeon pea (*Cajanus cajan* L. Millsp) from mature embryonal axes. *Plant Growth Regulation* 30:31-36. doi: 10.1023/A: 1006394402210.

Geetha, N., Venkatachalam, P., Prakash, V. and Lakshmi, G. 1998. High frequency induction of multiple shoots and plant regeneration from seedling explants of pigeon pea (*Cajanus cajan* L.) *Current Science* 75 (10):1036-1041.

Guo, B., Abbasi, B.H., Zeb, A., Xu, L.L. and Wei, Y.H. 2013. Thidiazuron: a multi-dimensional plant growth regulator. *African Journal of Biotechnology* 10(45): 8984-9000.

Heisler, M.G., Ohno, C., Das, P., Sieber, P., Reddy G.V., Long, J.A. and Meyerowitz, E.M. 2005. Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. *Current Biol*ology 15:1899-1911.

Hnatuszko-Konka, K., Kowalczyk, T., Gerszberg, A., Wiktorek-Smagur, A. and Kononowicz, A. K. 2014. *Phaseolus vulgaris*-Recalcitrant potential. *Biotechnology advances* 32(7): 1205-1215.

Hochholdinger, F. and Zimmermann, R. 2008. Conserved and diverse mechanisms in root development. *Current Opinion in Plant Biology* 11: 70-74.

Ikeuchi, M., Sugimoto, K. and Iwase, A. 2013. Plant callus: Mechanisms of induction and repression. *The Plant Cell* 25(9): 3159-3173.

Kaoneka, S.R., Saxena, R.K., Silim, S.N., Odeny, D.A., Ganga Rao, N.V.P.R., Shimelis, H.A., Siambi, M. and Varshney, R.K. 2016. Pigeon pea breeding in eastern and southern Africa: Challenges and opportunities. *Plant Breeding* 135: 148-154. doi: 10.1111/pbr.12340

Karimi, R., Owuoche, J.O. and Silim, S.N. 2010. Inheritance of *Fusarium* wilt resistance in pigeon pea [*Cajanus cajan* (L.) Mill spaugh]. *The Indian Journal of Genetics and Plant Breeding* 70(3): 271-276.

Krishna, G., Reddy, P. S., Ramteke, P. W., Rambabu, P., Tawar, K.B. and Bhattacharya, P. 2011a. *Agrobacterium*-mediated genetic transformation of pigeon pea [*Cajanus cajan* (L.) Millsp.] for resistance to legume pod borer *Helicoverpa armigera*. *Journal of Crop Science and Biotechnology* 14(3):197-204.

Krishna, G., Reddy, P., Pramod, W., Rambabu, P., Sayed, S., Rana, D. and Bhattacharya, P. 2011b. *In vitro* regeneration through organogenesis and somatic embryogenesis in pigeon pea [*Cajanus cajan* (L.) Millsp.] cv. JKR105. *Physiology and Molecular Biology of Plants* 17(4): 375–385. doi: 10.1007/s12298-011-0079-1.

Lateef, R.S. and Reed, W. 1983. Review of crop losses caused by insect pests in pigeon pea internationally and in India. *Indian Journal of Entomology* 45:284 -293

Ljung, K., Bhalerao, R.P., and Sandberg, G. 2001. Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. *Plant Journal* 28:465-474.

Mano, Y and Nemoto, K. 2012. The pathway of auxin biosynthesis in plants. *Journal of Experimental Botany* 1-20. doi:10.1093/jxb/ers091.

Mergeai, G., Kimani, P., Mwang'ombe, A., Olubayo, F., Smith, C., Audi, P., Baudoin, J. and Roi, A. 2001. Survey of pigeon pea production systems. Utilisation and marketing in semi-arid lands of Kenya. *Biotechnology, Agronomy, Society*, *Environment* 5(3):145-153.

Mohan, M.L. and Krishnamurthy, K.V. 2002. Somatic embryogenesis and plant regeneration in pigeonpea. *Journal of Plant Biology* 45(1):19-25.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Journal of Plant Physiology* 15: 473- 497.

Nalini, M., Reena, M.J.T., Sastri, D.C. and Moss, J.P. 1996. Somatic embryogenesis in pigeon pea (*Cajanus cajan* L. Millsp.). *Indian Journal of Experimental Biology* 34:282-284.

Nene, Y.L. and Sheila, V.K. 1990. The pigeon pea, CAB International, Wellingford, UK. pp. 1-14.

Omanga, P.A. 1997. Improvement of pigeon pea and cowpea for drought, disease and insect pest tolerance through induced mutations (IAEA-TECDOC-951). International Atomic Energy Agency (IAEA). http://www.iaea.org/inis/collection/NCLCollectionStore/_Public/28/054/28054954.pdf

Overvoorde, P., Fukaki, H. and Beeckman, T. 2010. Auxin control of root development. *Cold Spring Harbor Perspectives in Biology* 2(6) doi: 10.1101/cshperspect.a001537.

Pambo, K.O. 2014. Application of the commodity approach to pigeonpea value-chain analysis in Kenya. A paper submitted for presentation at the FAO Hosted Session at the IFAMA World Forum 2014 Cape Town, South Africa, June 18, 2014. http://purl.umn.edu/173485

Parde, V.D., Sharma, H. C. and Kachole, M.S. 2012. Protease inhibitors in wild relatives of pigeon pea against the cotton bollworm/legume pod borer, *Helicoverpa armigera*. *American Journal of Plant Sciences* 3:627.

Prasad, M.G., Prasad, T.N.V. K.V. and Sudhakar, P. 2011. In vitro proliferation of shoot regeneration from embryo of *Cajanus cajan* L. (var.LGG-29). *Journal of Developmental Biology and Tissue Engineering* 3 (5): 62-65.

Rotimi, J. and Iloba, B.N. 2008. Assessment of yield losses in tall varieties of pigeon peas due to the flower thrips, *Megalurothrips sjostedti* Trybom. (Thysanoptera: Thripi dae). *Bioscience Research Communication* 20:305-308.

Scarpella, E., Barkoulas, M. and Tsiantis, M. 2010. Control of leaf and vein development by auxin. *Cold Spring Harbor Perspectives in Biology* 2(1):a001511.

Sharma, O.P., Gopali, J.B., Yelshetty, S., Bambawale, O.M., Garg, D.K. and Bhosle, B.B. 2010. Pests of pigeon pea and their Management, NCIPM, LBS Building, IARI Campus, New Delhi-110012, India.

Sreenivasu, K., Malik, S.K., Kumar, P. and Sharma, R.P. 1998. Plant regeneration via somatic embryogenesis in Pigeon pea (*Cajanus cajan* L.Millsp). *Plant Cell Reports* 17: 294-297

Srivastava, J. 2013. *In vitro* regeneration protocol for pigeon pea-a review. *International Journal of Food, Agriculture and Veterinary Sciences* 3 (2):63.

Thorpe, T.A., Stasolla, C., Yeung, E.C., de Klerk, G.J., Roberts, A.V. and George, E.F. 2008. Plant propagation by tissue culture. The background. The components of plant tissue culture media II: Organic additions, osmotic and pH effects and support systems. *Springer Netherlands Dordrecht* (1):115-173.

Varshney, R.K., Penmetsa, R.V., Dutta, S., Kulwal, P.L., Saxena, R.K., Datta, S., Sharma, T.R, Rosen, B., Carrasquilla-Garcia, N., Farmer, A.D., Dubey, A., Saxena, K.B., Gao, J., Fakrudin, B., Singh, M.N., Singh, B.P., Wanjari, K.B., Yuan, M., Srivastava, R.K., Kilian, A., Upadhyaya, H.D., Mallikarjuna, N., Town, C.D., Bruening, G.E., He, G., May, G.D., McCombie, R., Jackson, S.A., Singh, NK. and Cook, D.R. 2010. Pigeon pea genomics initiative (PGI): An international effort to improve crop productivity of pigeon pea (*Cajanus cajan* L.) *Molecular Breeding*; 26:393-408. doi: 10. 1007/s11032-009-9327-2.

Veltcheva, M., Svetleva, D., Petkova, S. and Perl, A. 2005. *In vitro* regeneration and genetic transformationof common bean (*Phaseolus vulgaris* L.): Problems and progress. *Scientia Horticulturae* 107: 2-10.