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Regeneration and Transformation of Common Bean (*Phaseolus vulgaris* L.)

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REGENERATION AND TRANSFORMATION OF COMMON BEAN (*Phaseolus vulgaris* L.)

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Common bean is the most important grain legume for direct human consumption and serves as main source of dietary protein for millions of people in developing world. Genetic transformation methods can serve as an important tool to complement traditional plant breeding methods for common bean improvement. Low transformation frequencies and unstable genetic integration associated with biolistic methods limits its use for routine transformation of common bean. In an attempt to develop *Agrobacterium tumefaciens* mediated transformation protocol for common bean, potential of primary leaves as an explant was analyzed following unsuccessful results with other tissues. Primary leaf explants were prepared from 5 days old seedlings of common bean great northern cultivar Coyne and inoculated with *Agrobacterium tumefaciens* strain EHA 101. Explants were cultured on MSB5 media with two different concentrations of growth hormones. Primary leaf explants show significant differences in growth on these two different media after 2 weeks and 4 weeks of culture. Transgenic callus and fully transformed roots were recovered for the first time using primary leaf explants. In attempt to regenerate primary leaf explants different factors were analyzed. Four different predefined major and minor salt compositions were tested for their suitability for leaf explant regeneration. Leaf explants exhibit significant differences in growth on four different media, indicating possible role of total nitrogen and

NH_4^+:NO_3^- ratio on the growth of leaf explants. This work suggests that leaf explants can be pursued for common bean transformation.

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Chapter 1

Introduction

Common bean is the most important grain legume for direct human consumption in the world and are major staple of Eastern and Southern Africa. Common beans are an important source of dietary protein (20-25% of seed weight) and complements cereals for over half a billion people in Latin America (Gepts, 2001; Graham & Ranalli, 1997). In addition to proteins, common beans are an important source of iron, phosphorus, magnesium, manganese and in lesser degree zinc, copper and calcium. At an average consumption level of 15-20 kg per year per person beans provide 15-20% of adult requirement for a number of nutrients (Broughton et al. 2003). Common bean yields range from less than 500kg ha^{-1} in parts of Latin America and Africa to as high as 5000 kg ha^{-1} under experimental trials (Graham & Ranalli, 1997). Several factors like diseases, insect pests, lack of tolerant varieties to abiotic stresses like drought and marginal soils contributes to reduced yields. Different genes conferring tolerance or resistance to various diseases, insect pests as well as to different abiotic stresses are present in alien germplasm. For example, *Phaseolus polyanthus* is a source of resistance to Aschochyta blight (*Aschochyta rabiei)* and Bean Golden Yellow Mosaic Virus (BGYMV), *P.coccineus* is a source of resistance to BGYMV, anthracnose (*Colletotrichum lindemuthianum),* root rot and white mold (*Sclerotinia sclerotiorum), P. accutifolius* is a source of resistance to leaf hoppers (*Empoasca kraemeri* and *E. fabae* Harris) (Broughton et al. 2003; Singh & Schwartz. 2010). But imperfect chromosome pairing, male sterile F1 hybrids, difficulties in exploiting amphidiploids, inverse correlations among useful traits

and requirement of embryo rescue methods to get hybrids, hinders the efforts to transfer important traits from related species to common bean (Veltcheva et al. 2005). Due to these obstacles less than 5% of available genetic diversity has been used globally in commercial cultivars (Broughton et al 2003). Genetic transformation can serve as an important tool to complement traditional breeding programs for crop improvement by overcoming these limitations (Mukeshimana et al. 2013). Throughout the world, regular efforts are being made to develop efficient transformation protocol for common bean. Nevertheless, till date very limited success has been achieved in common bean transformation efforts.

Stable transformation of common bean has been achieved using direct gene transfer methods such as particle bombardment and electroporation, but with very low transformation frequencies 0.03% (Russell et al. 1993), 0.9% (Aragao et al. 1996), 0.8 % (Vianna et al. 2004) and 0.66% (Bonfim et al. 2007). Along with low frequencies, high copy number of inserts, complex integration patterns, higher chances of chimeric plants and high costs make this approach less attractive compared to *Agrobacterium* mediated transformation methods (Jackson et al. 2013).

On the other hand, common bean transformation efforts using *Agrobacterium* mediated gene transfer has not been successful. Genga et al. (1990) transformed cotyledonary node and primary leaf explants with different *Agrobacterium* strains and were able to produce callus on kanamycin selection media but failed to get full explants. McClean et al. (1991) transformed cotyledons and hypocotyls using *A. tumefaciens* strain

C58Z707/pGA482 and *A.rhizogenes* strain A4RS respectively, but they were not successful in regeneration of full plants. Franklin et al. (1993) produced transformed GUS positive callus from inoculation of kidney bean with *A.tumefaciens* strain EHA 101. Lewis and Bliss (1994) transformed meristematic regions of different shoot types using stab inoculation with C58 strain, but failed to regenerate any shoots from transformed cells. Kapila et al. (1997) transformed common bean intact leaves through vacuum infiltration of *Agrobacterium* for transient gene expression studies and found high GUS expression of leaf explants with 20-90% of leaf area showing GUS expression. More recently, Mukeshimana et al. (2013) attempted common bean transformation using embryo axis explants. However, they got chimeric plants, which failed to acclimatize in soil.

Several reports are available for whole plant regeneration in common bean using shoot apex cultures (Kartha et al. 1981; Martins et al. 1984), cotyledonary nodes (Mohamed et al. 1992; Tháo et al. 2013), cotyledonary node devoid of axillary buds (McClean et al. 1989; Mariotti et al. 1989), cotyledonary node with axillary buds (Franklin et al. 1991), embryo axis (P. Delgado-Sánchez et al. 2006) embryo derived callus (Zambre et al. 1998). But no reproducible confirmed reports of *Agrobacterium tumefaciens* mediated transformation using these regeneration protocols is available till date.

Hence, the objective of this research was to (i) find a suitable explant for *Agrobacterium tumefaciens* mediated transformation of common bean (ii) to attempt the regeneration of suitable explant.

Literature Review

Common bean

Common bean (*Phaseolus vulgaris* L.) is a self-pollinated, diploid (2x=2n=22) plant that belongs to genus *Phaseolus* in family Leguminosae and is the most important species of the genus economically and scientifically (Gepts, 2001). Common beans are grown on all continents except Antarctica (Gepts, 1998) in wide range of climatic conditions ranging from humid tropics in Latin America and Africa to semi-arid highlands of Mexico and the high plains of US and Canada (Graham & Ranalli, 1997).

Common beans are the most important grain legume for direct human consumption (Broughton et al., 2003; Gepts, 2001; Graham & Ranalli, 1997). They are an important source of dietary protein (20-25% of seed weight) (Broughton et al., 2003;Miklas & Singh, 2007; Steel et al., 1995) and serve as main staple food in Eastern and Southern Africa. In Latin America, common beans complement cereals for over half a billion people. Along with proteins, common beans are an important source of iron, phosphorus, magnesium, manganese, and in lesser degree of zinc, copper and calcium (Broughton et al., 2003).

Common bean production spans from 52^0N to 32^0S latitude and from near sea level to elevation of more than 3000m (Graham & Ranalli, 1997). Worldwide common beans are grown on more than 14 million hectares (Miklas & Singh, 2007). 54.5% of total production was from Americas (7.7million metric tons) and Africa (5.9 million metric

tons), while remaining 45.5 % production comes of Asia, Europe and Oceania (FAOSTAT, 2014). Millions of small-scale farmers in Latin America and Africa rely on the production and sale of beans as an important source of household income. Beans are consumed as mature grain, immature seed and green pods (Broughton et al. 2003; Gepts, 2001; Graham & Ranalli, 1997).

Reasons for low yields of common bean

In Latin America and Africa, although common beans served as main source of dietary proteins, and grown on large acreage, but crop yields in developing countries ranges around 1035 kg ha^{-1} with yields as low as 638 kg ha^{-1} in Uganda and 671 kg ha^{-1} in Rwanda, which are very low compared to developed countries where average yields are around 1944 kg ha^{-1} (Gepts et al. 2008). Common beans are generally grown by resource poor farmers on small and marginal lands. In Latin America 80% of bean production is done on 1ha to 10 ha size of farms. In Africa beans are grown by small farmers especially by women farmers, on small farms with minimal inputs (Broughton et al. 2003). Beans grown by these resource poor farmers are more vulnerable to diseases, insect pests and abiotic stresses.

Among diseases, the major constraints in bean production are bean anthracnose (*Colletotrichum lindemuthianum*), angular leaf spot (*Phaeoisariopsis griseola),* halo blight (Pseudomonas *phaseolicola),* rust (*Uromyces phaseoli),* and *bean common mosaic virus*. On average 20-100% yield loss occurs due to various diseases in common bean (S. P. Singh & H. F. Schwartz, 2010). Various insect pests like leafhopper (*Empoasca*

kraemeri and *Empoasca fabae* Harris), thrips (*Thrips palmi)*,bean pod weevil (*Apion godmani* and *Apion aurichalceum* Wagner), whitefly (*Bemisia tabaci)*, bean fly (*Ophiomyia phaseoli* Tryon), aphids (e.g., *Aphis fabae* Scopoli), chrysomelids (*Ootheca species*), pod borer [*Maruca testulalis* (Geyer)], and mites [*Tetranychus cinnabarinus* Boisd*., Polyphagotarsonemus latus* (Banks) affect common beans leading 35% to 100% yield losses (S. Singh & H. Schwartz, 2010). Sixty percent of bean production in developing world occurs under conditions of significant drought stress, making it the second most important constraint in bean production after diseases (Schneider et al. 1997). Significant flower and pod abortion occurs if drought occurs at flowering and pod setting time (Graham & Ranalli, 1997). In developing countries nutrient deficient soils adds up the yield losses. According to Broughton et al. (2003) around 50% of beans in Latin America and 75% in Africa are grown on soils deficient in phosphorus. All these factors contribute towards reduced yields of common bean in these areas.

Need for a transformation system for common bean

Progress in conventional genetic and breeding improvement of beans was hampered in many aspects (Veltcheva et al. 2005). Domestication and selection results in loss of genetic diversity (Beaver & Osorno, 2009) of common bean. Potential incompatibility of Andean and Mesoamerican germplasm leads to F_1 hybrid weakness in hybridization between plants from these two gene pools (Kelly et al. 1998). Several species of *Phaseolus* can be hybridized to common bean, though the hybrid seeds are only likely to survive when embryo cultured on synthetic media (Graham & Ranalli, 1997). Even F1 hybrids obtained through embryo rescue technique from crosses between

P.vulgaris and other *Phaseolus* species like *P.filiformis, P.angustissimus, P.lunatus*, produce sterile plants (Broughton et al., 2003). All these obstacles limit the use of conventional breeding methods and make it more tedious and time-consuming process. Plant genetic engineering methods and systems offer to overcome these limitations (Veltcheva et al., 2005) and work as an extension to conventional plant breeding methods (Svetleva et al. 2003).

Common bean transformation using direct gene transfer methods

Stable transgenic common bean plants were obtained by using particle bombardment techniques. Electric discharged particle acceleration technology was used to transform navy bean cultivar Seafarer meristems with a very low frequency of 0.03% of germline transformed plants (Russell et al. 1993). Aragão et al. (1996) used particle bombardment to deliver DNA into embryonic axis of common bean and recovered transgenic plants with an average frequency of 0.9%. Later the same method as described by Aragão et al. (1996) was used by other groups (Bonfim et al. 2007; Vianna et al., 2004) and reported almost similar transformation frequencies of 0.7-0.8% and 0.66% respectively. Rech et al. (2008) reported increased transformation frequency of 2.7% by particle bombardment of common bean and using herbicide imazapyr as selection agent. But particle bombardment results multicopy insertions and complex integration events (Jackson et al. 2013). Moreover, very low transformation frequencies make recovery of large number of independently derived stable transformation events labor intensive and rather expensive (Christou, 1992). Insertion of multiple gene copies may also lead to co-

suppression and gene silencing. In contrast, transgenic plants from *Agrobacterium* mediated transformation have better chances to get intact copy of transgenes, better fertility than particle bombardment (Dai et al., 2001). These features make *Agrobacterium* mediated transformation the first choice of the scientific community.

Agrobacterium **mediated transformation of common bean**

Agrobacterium tumefaciens mediated transformation of common bean is still lacking an efficient protocol. McClean et al. (1991) transformed cotyledons and hypocotyls using *A.tumefaciens* strain C58Z707/pGA482 and *A.rhizogenes* strain A4RS respectively and managed to get callus but failed to regenerate plants. GUS positive callus from kidney beans was obtained by inoculating with *A.tumefaciens* strain EHA 101 (Franklin et al. 1993). Different shoot types were transformed through stab inoculating with *A.tumefaciens* strain C58, but no shoots were recovered from transgenic cells (Lewis & Bliss, 1994). Mariotti et al. (1989) reported putative transformation of common bean, but no molecular evidence of transformation or data on transmission of transgene to the next generation was presented. Mukeshimana et al. (2013) tried *Agrobacterium tumefaciens* mediated transformation using 3 strains GV3101, LBA4404 and EHA 105 with four common bean cultivars. They were able to get plantlets after 6 weeks but these plantlets failed to develop into normal plants. Collado et al. (2015) reported transformation of *Phaseolus vulgaris* cv. CIAP7247F via *Agrobacterium tumefaciens* using indirect organogenesis. Reported transformation efficiency was 2.8%. This new method is yet to be duplicated in other labs for repeatability and effectiveness.

Agrobacterium mediated transformation of other *Phaseolus* species

Although common bean transformation with *Agrobacterium* was still not possible, other grain legumes and species of *Phaseolus* were transformed successfully through *Agrobacterium.* Dillen et al. (1997) reported *Agrobacterium* mediated transformation of *Phaseolus acutifolius* A.Gray (tepary bean) inoculating callus derived from bud explants with *Agrobacterium tumefaciens* C58C1Rif^{R}(pMP90) strain. Transgenic plants were recovered and transmission of transgene to progeny was analyzed. De Clercq et al. (2002) reported improved transformation of *P.acutifolius* A.Gray following the procedure described by Dillen et al. (1997). Zambre et al. (2005) also reported transformation of cultivated genotypes of *P.acutifolius* A.Gray.

Agrobacterium mediated transformation of other legume species

Successful transformation reports for other grain legumes like *Glycine max* (Chee et al. 1989; Hinchee et al., 1988; Wang & Xu, 2008; Zhang et al. 1999), *Arachis hypogaea* L. (Rohini & Rao, 2001; Tiwari & Tuli, 2012), *Cicer arietinum* ((Bhattacharjee et al. 2010; Khatodia et al. 2014), Lentil (Akcay et al. 2009; Das et al. 2012), *Vigna ungiculata* L. (Citadin, Cruz, & Aragão, 2013; Popelka et al. 2006), *Vigna mungo* L.Hepper (Saini et al. 2003; Saini & Jaiwal, 2005), *Vigna angularis* (Yamada et al. 2001) , *Pisum sativum* L. (Pniewski & Kapusta, 2005; Schroeder et al. 1993; Svabova et al. 2005), *Vicia faba* L. (Hanafy et al. 2005) , *Vigna radiata* L. Wilczek (Mahalakshmi et al., 2006) using *Agrobacterium tumefaciens* mediated gene transfer are available.

Common bean regeneration

Various protocols are available based on direct organogenesis or shoot development from meristem cells for common bean regeneration. Full plants from common bean cultivar Fonix and Maxidor using intact seedling and cotyledonary node explants were regenerated (Ahmed et al. 2002; Dang and Wei 2009; Tháo et al. 2013). Whole plants were also regenerated using embryonic axis explants (Delgado-Sánchez et al. 2006; Castillo et al., 2015; Quintero-Jimenez et al., 2010). Veltcheva et al. (2005) regenerated plants from leaf petioles of three Bulgarian common bean varieties and reported genotype dependent reactions. Sabzikar et al. (2010) used apical meristems as explant for regeneration studies of 10 common bean cultivars and observed that race of cultivar plays important role in apical shoot meristem multiplication. Arellano et al. (2009) regenerated 10 different common bean cultivars using apical meristem and cotyledonary node explants through indirect organogenesis. But the frequency of regeneration was very low.

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Chapter 2

Regeneration and transformation of common bean (*Phaseolus vulgaris* L.) using cotyledonary node and embryonic axis explants

Abstract

An efficient protocol of common bean transformation using *Agrobacterium tumefaciens* is still unavailable. Several regeneration reports are available using cotyledonary node or embryonic axis as the starting explants. These explants were used for direct organogenesis exploiting the meristems for direct shoot production. Attempts were made to transform cotyledonary node explants and embryonic axes explants through *Agrobacterium tumefaciens* mediated gene transfer. Cotyledonary node and embryonic axis explants were prepared from 5 day germinated seedlings and inoculated with *Agrobacterium tumefaciens* strain EHA 101. Cotyledonary node and embryonic axes explants did not yield any GUS positive shoots after culturing on selection media. Transient GUS expression studies suggest that for these explants lack of *Agrobacterium* infection in the meristematic region might be the reason for non-production of transgenic shoots/plants.

Keywords: Common bean; *Agrobacterium tumefaciens*; cotyledonary node; embryonic axis transformation; regeneration

Introduction

Common bean is the most important grain legume for direct human consumption (Gepts et al. 2008; Broughton et al. 2003). It is a major source of protein in the developing world and provides about 30% of protein intake day^{-1} (Cabrera-Ponce et al. 2015). Crop improvement by introducing desirable traits like nutritional quality improvement and, drought resistance through genetic transformation is of considerable interest (Aragao et al. 1997). Common bean transformation has been achieved with a limited success through particle acceleration methods but with very low transformation frequencies ranging from 0.03% to 0.9% (Russell et al. 1993; Aragao et al. 1996; Vianna et al. 2004; Bonfim et al. 2007). Due to several benefits like low copy number, stable integration patterns over biolistic methods, Agrobacterium mediated transformation is favored for routine and efficient transformation of plants (Jackson et al. 2013).

Agrobacterium mediated transformation of common bean is still lacking a successful protocol. Various attempts has been made using different starting explants for common bean transformation through *Agrobacterium tumefaciens* e.g. embryonic axes (Mukeshimana et al. 2013; Amugune et al. 2011) cotyledoanry nodes (McClean et al. 1991; Genga et al. 1990), leaf discs and hypocotyls (Franklin et al. 1993). All these attempts were unsuccessful in attaining the transgenic plants.

Various reports are available for regeneration of whole plants from cotyledonary node explants (Dang and Wei., 2009; Tháo et al. 2013), embryonic axes (Sanchez et al. 2005; Kwapta et al. 2010; Quintero-Jimenez et al., 2010; Castillo et al., 2015), embryo

derived callus (Zambre et al. 1998) in common bean. These reports suggest that it is possible to regenerate common bean, which was considered recalcitrant towards *in vitro* regeneration.

Although for common bean, till date no successful transformation using *Agrobacterium tumefaciens* gene transfer method combined with direct organogenesis is available. But in other legume crops direct organogenesis is successfully compiled into *Agrobacterium tumefaciens* mediated transformation protocols e.g. in soybean (Zhang et al. 1999, Olhoft, Paula M., et al 2003), peanut (Anuradha et al. 2006).

As a first step towards transformation of common bean through *Agrobacterium tumefaciens* mediated gene delivery method, embryonic axis and cotyledonary node explants were tried following the soybean transformation protocol using cotyledonary node explants (Zhang et al. 1999).

Material and methods

Plant material

Seeds of *P. vulgaris* L. great northern cultivar "Coyne" (Urrea et al. 2009) were provided by Dr. Carlos Urrea, Associate Professor, Agronomy and Horticulture University of Nebraska-Lincoln. Seeds were surface sterilized by an overnight exposure to chlorine gas (Di et al. 1996). Sterilized seeds were germinated, in 100 X 20 mm Petri dishes, on germination media (GM) containing Gamborg's B5 inorganic salts and vitamins (Gamborg et al. 1968), 2%sucrose, 0.8% agar and 1mg l^{-1} BAP. pH was adjusted to 5.6 before autoclaving for 21minutes at 121^0C and 1.07kg cm^{-2}. The plates were stacked 5 high and placed in plastic bags in which 4, approx. 3 inch, slits were made with scissors. Seeds were germinated for 5 days in a growth room, at 24^0 C, 18/6 light regime, under a light intensity of approximately 150 μmol $s^{-1}m^{-2}$.

Explant preparation

Cotyledonary node explants

Cotyledonary node explants were prepared from 5 day old seedlings after removing the seed coat and radicle and cutting epicotyl and hypocotyls approximately 2 mm above and below the nodal region. Then two explants from one seedling were made by longitudinally cutting the embryonic axis part into halves. Meristematic region present in the nodal region was macerated by 7-10 shallow diagonal cuts using a sterilized scalpel with No. 11 blade. These explants were then inoculated with *Agrobacterium* inoculum.

For pre-culture studies, cotyledonary node explants were prepared as described above except macerating the nodal region, from 5 day old seedlings and pre-cultured on MSB5 (Murashige and Skoog's major and minor salts with B5 vitamins) media with 3% sucrose, pH 5.7 amended with 2.5mg l^{-1} BAP and 0.1mg l^{-1} NAA, solidified with 0.8% purified agar. Five explants per petri plate were placed by immersing hypocotyl end into media and adaxial side upwards. Explants were cultured for 1 week under 18 hour light at 24^0C. After 1 week, wounding was made by making shallow cuts around the initiated growth in nodal region and used for inoculation.

Embryo axis

To prepare embryo axis explants cotyledons were removed from the 5 day old seedlings carefully keeping the axillary nodes intact. Hypocotyl and primary leaves were cut from the explant and resulting explant was bisected along the longitudinal plane making two explants from one seeding. These final explants were inoculated by immersing in the *Agrobacterium* inoculum.

Agrobacterium strains

Agrobacterium tumefaciens strain EHA 101 (Hood et al. 1986) harboring binary plasmid pPTN 1043 or pPTN 289 was used for inoculation. pPTN 1043 contains the *neomycin phosphotransferase* gene (*npt*II) driven by cauliflower mosaic virus 35S promoter and an intron interrupted *β-glucronidase* gene (*gus*A) which is controlled by 35S promoter (Figure 2.1.a). Binary plasmid pPTN 289 contains *bar* gene as selection marker driven by *nopaline synthase (nos)* gene promoter and intron interrupted *gus*A as reporter gene driven by 35S promoter. (Figure 2.1. b). Single colonies of each strain were streaked on

plates with 25 ml of Luria Bertani (LB) media containing 25mg l^{-1} of each kanamycin, streptomycin, spectinomycin and chloramphinol antibiotics and incubated in 28^0C for 2 days. Day before inoculation *Agrobacterium* cultures from these plates were grown in 5 ml YEP medium (10 g l^{-1} peptone, 5 g l^{-1} yeast extract and 5 g l^{-1} NaCl, pH 7.0) amended with appropriate antibiotics to an OD_{650} = 0.5 to 1.0 at 28^0C. Bacterial cultures were centrifuged at 3500 rpm for 10 minutes and the pellets re-suspended in to final OD_{650} = 0.5 to 1.0 in liquid co-culture media which was 1/10 Gamborg's B5 media with 3% sucrose, 200µM acetosyringone and supplemented with 1.67 mg l^{-1} BAP for cotyledonary node explants, 2.5 mg l^{-1} BAP with 0.1 mg l^{-1} NAA for pre-cultured cotyledonary node explants, and with 4 mg l^{-1} BAP, 0.1 mg l^{-1} IAA for embryonic axis explants. Media was buffered with 20mM MES and pH was adjusted to 5.4.

Plant transformation

Cotyledonary node explants were immersed in *Agrobacterium tumefaciens* EHA 101(pPTN 1043) or pPTN 289 inoculum for 30 minutes and then co-cultured on 100 X 15 mm petri plates containing 4 pieces of Whattman #1 filter paper immersed in 5 ml of liquid co-culture media which is 1/10 Gamborg's B5 media with 200µM acetosyringone amended with 1.67 mg l^{-1} BAP. Pre-cultured cotyledonary node explants were co-cultured in 1/10 MSB5 media with 200µM acetosyringone amended with 2.5 mg l^{-1} BAP, 0.1 mg l^{-1} NAA. Cotyledonary node explants were cultured by placing the nodal region in touch with the filter paper. Explants were co-cultivated for 3 days at 24^0 C, 18/6 light regime, under a light intensity of approximately 80 μmol s^{-1} m^{-2}, after wrapping the petri

plates with parafilm. Following the co-cultivation period explants were briefly washed in B5 media supplemented with 3% sucrose, 75 mg l^{-1} of each ticarcillin, cefotaxime and vancomycin, buffered with 3mM MES and pH adjusted to 5.7 along with 1.67 mg l^{-1} BAP. Pre-cultured cotyledonary node explants were washed in MSB5 media supplemented with 3% sucrose, 75 mg l^{-1} of each ticarcillin, cefotaxime and vancomycin, buffered with 3mM MES and pH adjusted to 5.7 along with 2.5 mg l^{-1} BAP, 0.1 mg l^{-1} NAA (referred as SIM62 wash media). Growth regulators, vitamins and antibiotics were filter sterilized and added to media post autoclaving. After washing, explants were cultured (5 per plate) in 100 X 20 mm petri plates, adaxial side up with the hypocotyl imbedded in the medium, containing wash media solidified by 0.8% purified agar and amended with 10mg/l G418 (referred as SIM63) or in SIM62 for pre-cultured cotyledonary nodes. Plates were wrapped with 3M pressure sensitive tape and cultured at 24^0 C, 18/6 light regime, under a light intensity of approximately 150 μmol $s^{-1}m^{-2}$. After 2 weeks of culture, cotyledonary node explants showing the multiple shoot bud initiation or shoot growth were tested for histochemical GUS assay.

Embryo axis explants were inoculated in similar manner as described for cotyledonary node explants. Co-culture media for embryo axis explants was composed of 1/10 Gamborg's B5 media with 200µM acetosyringone amended with 4mg l^{-1} BAP and 0.1 mg l^{-1} IAA. Embryo axis explants were cultured by placing the cut side in touch with the filter paper. Explants were co-cultivated for 3 days at 24^0 C, 18/6 light regime, under a light intensity of approximately 80 μmol $s^{-1}m^{-2}$, after wrapping the petri plates with parafilm. Following the co-cultivation period explants were briefly washed in B5 media

supplemented with 3% sucrose, 75 mg l^{-1} of each ticarcillin, cefotaxime and vancomycin, buffered with 3mM MES and pH adjusted to 5.7 along with 4 mg l^{-1} BAP and 0.1 mg l^{-1} IAA. Growth regulators, vitamins and antibiotics were filter sterilized and added to media post autoclaving. After washing, explants were cultured (5 per plate) in 100 X 20 mm petri plates, containing wash media solidified by 0.8% purified agar and amended with either 0,10,15,20 mg l^{-1} G418 or 0,50,75,100 mg l^{-1} Kanamycin. Plates were wrapped with 3M pressure sensitive tape and cultured at 24^0 C, 18/6 light regime, under a light intensity of approximately 150 μmol $s^{-1}m^{-2}$.

After 2 weeks of culture, embryo axis explants showing growth were either transferred to fresh media with same level of selection or sacrificed for histochemical GUS assay.

Histochemical GUS assay

Histochemical GUS assay was performed as described by Zhang et al. (1999). Tissue were incubated submerged in X-GLUC, at 37^0C for 5-10 hours. The tissue was subsequently cleared in 70% ethanol prior to visualization. Any explant that has at least one blue spot was considered as GUS positive.

Results

Cotyledonary node explants

Common bean transformation efforts were initiated by following soybean transformation protocol (Zhang et al. 1999) in which cotyledonary nodes were used as an explant for *Agrobacterium tumefaciens* mediated transformation. After 2 weeks culture on SIM63, 45 out of 90 explants show growth of multiple shoot buds and 2-3 long shoots from the nodal area (Table 2.2). These were sacrificed for histochemical GUS assay.

During one week of pre-culture, cotyledonary node explants show initiation of growth from the nodal region. After 2 weeks culture on selection media, no explant show further growth from nodal region. They all turned brown and died.

Histochemical GUS assay

After 3 days of co-cultivation explants were analyzed for GUS expression. 30-50% of total tested explants show GUS expression (Table 2.1). The explants exhibit the GUS expression in cut hypocotyl end (Figure 2.2. (b)). No explant exhibit GUS expression in meristematic region.

Shoots along with multiple shoot buds growing on selection SIM63 were tested for GUS assay but no shoot showed GUS expression (Figure 2.3).

Transient GUS expression study was done for cotyledonary nodes inoculated after 1 week pre-culture on MSB5 media with 3% sucrose, pH 5.7, amended with 2.15mg/l BAP and 0.1mg/l NAA. In histochemical GUS assay done after 3 day co-cultivation, 30 out of

35 explants showed no GUS expression on any part of the cotyledonary node explant (Figure 2.4.(a)) (Table 2.3). No GUS expression was observed in the hypocotyl end, as in the case of explants without pre-culture. 5 explants show GUS expression on the new growth in the form of single small spot of blue color (Figure 2.4. (b)).

Embryonic axis

After 3 day co-cultivation, around 70-75% of embryonic axis explants showed GUS expression (Table2.4). GUS expression was mainly concentrated in hypocotyl and epicotyl region (Figure 2.5. a). After 4 weeks of culture on different levels of kanamycin selection and different G418 selection levels, all explant died. On kanamycin selection media explants died within first 2 weeks on all 3 levels, while on G418 media 12 out of 60 explants survived in first two weeks with 1-2 shoots growing from apical meristem on 10 mg l^{-1} G418, while on 15 mg l^{-1} and 20 mg l^{-1} G418 no explant survived (Table 2.5). When these growing shoots were tested for GUS assay, no shoot showed GUS expression (Figure 2.5. b). When sub-cultured on fresh media with 10 mg l^{-1} G428 selection, these explants died without any further growth.

Discussion

Direct organogenesis potential of explants having meristems like cotyledonary nodes and embryo axis has been successfully exploited for *Agrobacterium tumefaciens* mediated transformation of various legume crops. In our study, cotyledonary node explants lacked GUS expression in meristematic region, from which the new growth arises. This might be the reason for no regeneration on higher levels of selection and growth of non-transgenic shoots on lower selection levels. These experiments indicates that it's hard to infect meristematic parts of explants in common bean, supporting previous studies (Zhang et al. 1997; Mukeshimana et al. 2013). In other experiments, (data not shown) cotyledonary node explants were inoculated with pPTN 289 plasmid and cultured on media with 3 mg l^{-1} Glufosinate ammonium. No explant showed regeneration. When explants were cultured on 1 mg l^{-1} glufosinate ammonium some explants showed regeneration, which showed no GUS expression in new growth.

Preculturing of explants prior to *Agrobacterium* inoculation increases the *Agrobacterium* infection. In our study also 5 explants showed GUS expression in new growth, but this might be just the chimeric transformation. Also infecting the growing meristem with *Agrobacterium* leads to production of chimeric transformants.

Embryonic axes explants showed high GUS expression after 3 days of co-cultivation. Wounding throughout the explant occurred during handling and cutting the embryonic axes longitudinally may be the reason for high GUS expression. Generally GUS expression was concentrated in hypocotyl and epicotyl portions. Lack of

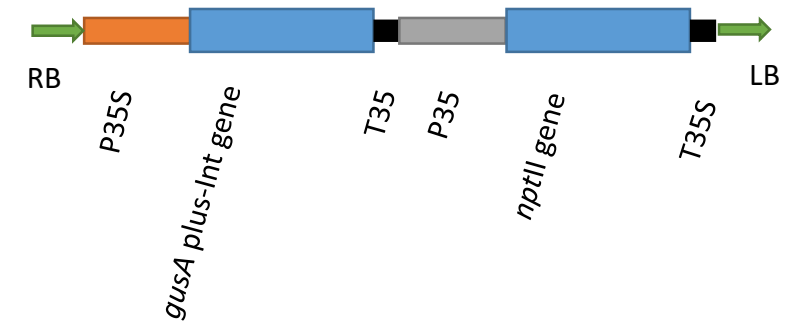
regeneration on selection media and production of non-transgenic growth during initial weeks of culture might be related to absence of *Agrobacterium infection* in apical meristem region. As reported by Aragao et al. (1997), morphological exposure of apical meristem of embryonic axis explants is genotype dependent and generally large apical meristem area is covered by primordial leaves and petiole of primary leaves making this area inaccessible. Also cutting the explant in half through apical meristem may cause damage to the apical meristem. Five different genotypes of common bean were tested for their response to *Agrobacterium tumefaciens* infection (data not given), and among those five genotypes great northern cultivar Coyne showed higher susceptibility compared to other genotypes. However, despite of higher susceptibility it was not possible to generate transgenic plants using cotyledonary node and embryonic axis explants. Other experiments were conducted using embryonic axis as explants and culturing them on different selection agents and different levels (data not given). On higher concentrations of selection agent these explants did not show regeneration, while on low concentrations they showed growth of non- transformed shoots.

Conclusion

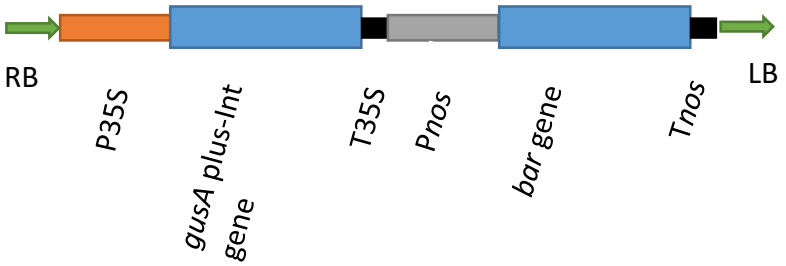
These results with use of embryo axis and cotyledonary node explants indicate that it is hard to transform meristematic region in these explants. More investigation is needed on part of morphological character of great northern culitvar Coyne. It was evident that pre-culturing cotyledonary node explants might increase the chances of *Agrobacterium*

infection in the nodal region, but further studies are needed on the age of seedling from which the explant is prepared and different pre-culture time periods.

Figures

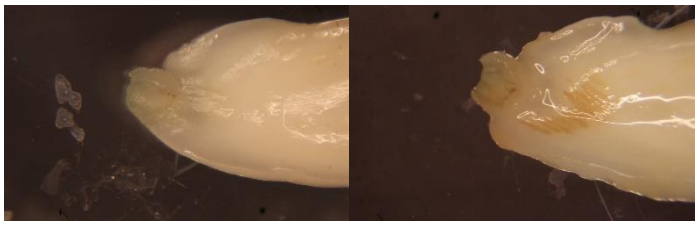


(a)

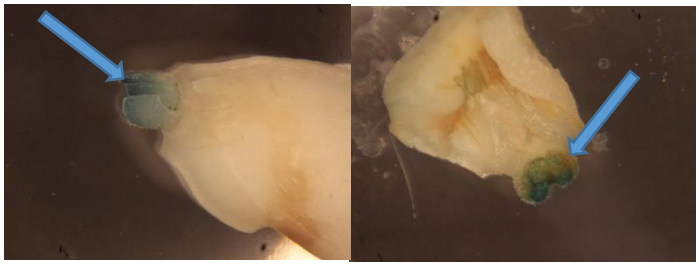


(b)

Figure 2.1. Schematic diagram of the T-DNA region from the binary vectors (a) pPTN 1043 and (b) pPTN 289. RB and LB, T-DNA right and left border sequences respectively; P35S, Cauliflower mosaic virus 35s promoter; *gusA* plus-int, β-*glucuronidase* gene interrupted by intron; T35S, cauliflower mosaic virus 35s polyA tail; *npt* II , *neomycin phosphotransferase* II conferring G418 resistance; P*nos* and T*nos*, *nopaline synthase* gene promoter and terminator respectively; *bar*, bialaphos resistance gene



(a)



(b)

Figure 2.2. GUS expression of cotyledonary node explants of common bean great northern cultivar ‘Coyne’ after 3 days of co-cultivation showing lack of GUS expression in meristematic area. Explants were inoculated with pPTN 1043 (a) explants showing no GUS expression (b) explants showing GUS expression at hypocotyl cut end (marked by arrow)

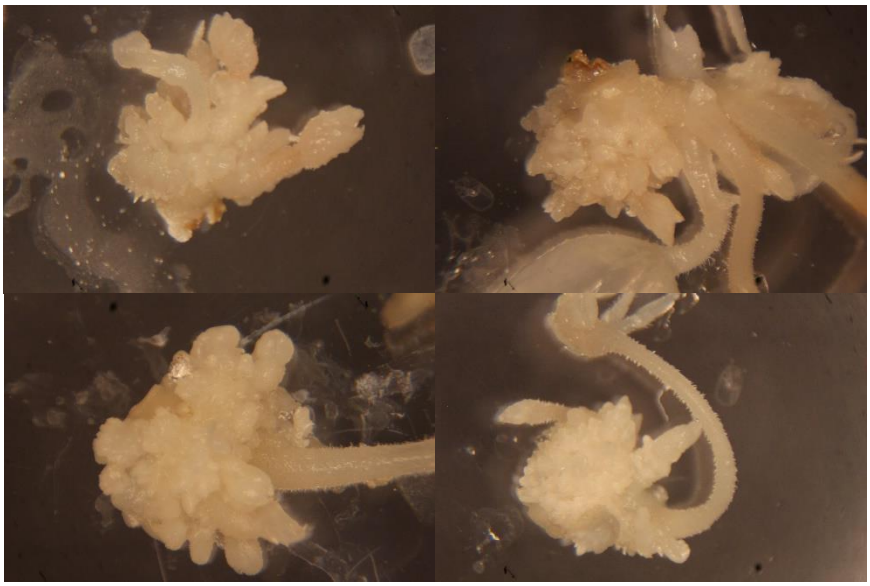
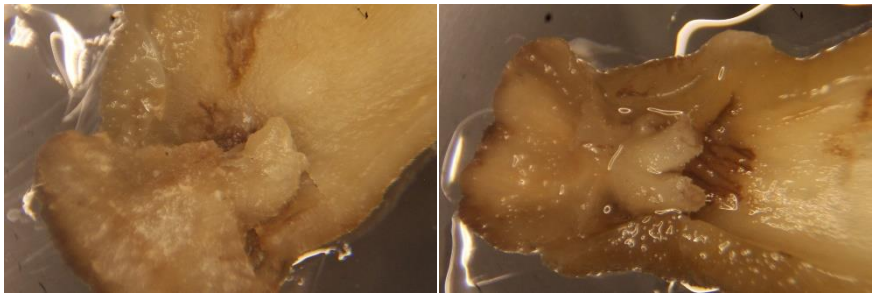
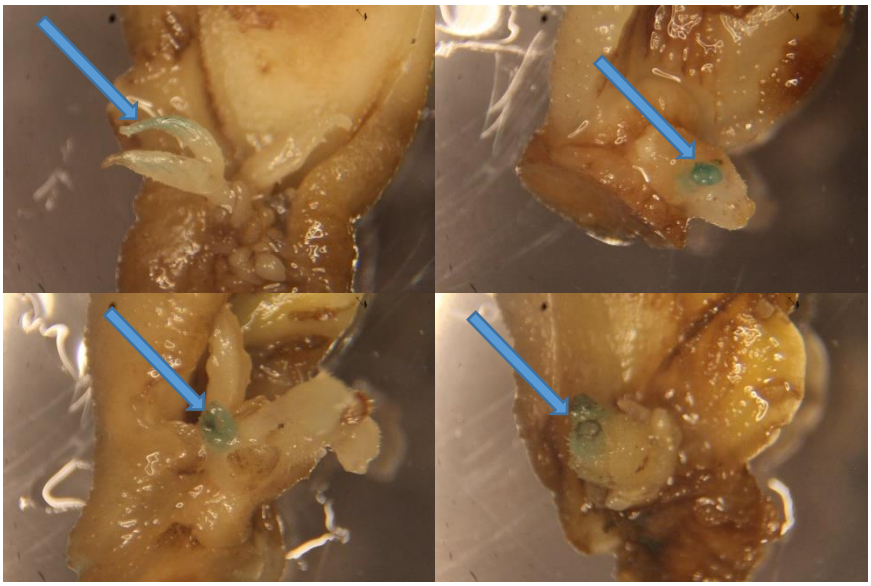


Figure 2.3. Histochemical GUS assay of coytledonary node explants of common bean great northern cultivar ‘Coyne’ after 2 weeks on culture media with 10 mg l^{-1} G418 selection

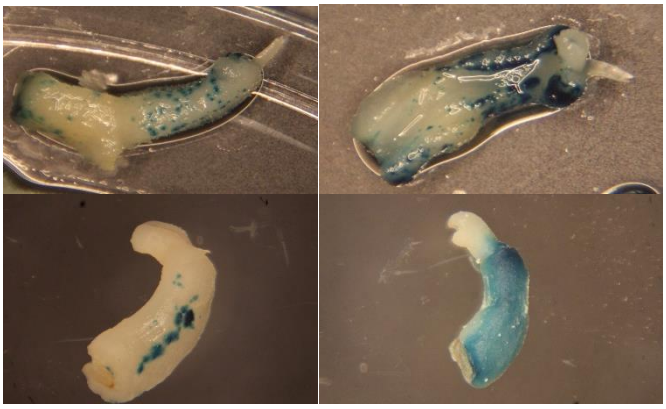


(a)

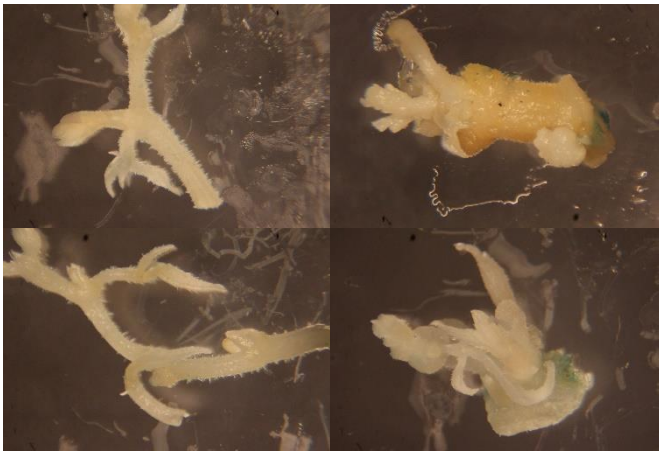


(b)

Figure 2.4. Histochemical GUS expression of cotyledonary node explants after 3 days of co-cultivation. Explants were prepared from 5 day germinated seeds and pre-cultured for 1 week on MSB5 media with 2.5 mg l^{-1} BAP (a) explants with no GUS expression (b) explants showing chimeric GUS expression at random points (pointed by arrows)



(a)



(b)

Figure 2.5. Histochemical GUS assay of embryonic axis explants of common bean great northern cultivar ‘Coyne’ (a) after 3 days of co-cultivation (b) after 2 weeks of growth on selection media EA2 supplemented with 10 mg l^{-1} G418

Tables

Table 2.1. Histochemical GUS expression of cotyledonary node explants after 3 days of co-cultivation on 10^{-1} B5 media with 3% sucrose, 20mM MES, 200µM acetosyringone, 1.67 mg l^{-1} BAP, pH 5.4

		Histochemical GUS assay response	
Experiment	Total number of explants tested for GUS	Explants with GUS expression at hypocotyl end (%)[a]	Explants with GUS expression at meristem part (%)
1	30	9 (30.0)	0
2	20	10(50.0)	0
Mean ± SE		9.5 ± 0.5	0 ± 0.0

[a] Percentage of GUS positive explants given in parenthesis = (number of GUS positive explants/total number of explants tested)*100

Table 2.2. Histochemical GUS assay of regenerated cotyledonary node explants after 2 weeks of culture on B5 media with 3% sucrose, 1.67 mg l^{-1} BAP, 10 mg l^{-1} G418 selection. Cotyledonary node explants were prepared from 5 days old seedlings and were inoculated with pPTN 1043

Experiment	Total number of explants	Explant with regeneration after 2 weeks of culture	Explants with GUS positive shoots
1	30	14	0
2	35	16	0
3	25	15	0
Total	90	45	0

Table 2.3. GUS expression of 1 week pre-cultured cotyledonary node explants after 3 day co-cultivation. Explants were prepared from 5 day old seedlings and pre-cultured for 1 week on MSB5 media with 3% sucrose, 2.5 mg l^{-1} BAP, 0.1 mg l^{-1} NAA and co-cultivated with pPTN 1043

Replication	Total number of explants tested	GUS positive explants	Efficiency (%)[a]
1	17	2	11.765
2	18	3	16.667
total	35	5	14.28(average)

[a] Efficiency (%) = (number of GUS positive explants/total number of explants tested)*100

Table 2.4. Histochemical GUS expression of embryonic axis explants after 3 days of co-cultivation on co-culture media 10^{-1} B5 media with 3% sucrose, 20mM MES, 200µM acetosyringone, 4 mg l^{-1} BAP, 0.1 mg l^{-1} IAA, pH 5.4

Experiment	Total explants tested for GUS	GUS positive explants	GUS positive explants (%)[a]
1	20	14	70
2	20	15	75
3	20	15	75
Mean ± SE		14.66 ± 0.33	71.66 (average)

[a] GUS positive explants(%) =(number of GUS positive explants/total number of explants tested)*100

Table 2.5. Embryonic axis regeneration after 2 and 4 weeks of culture on B5 media with 3% sucrose, 4 mg l^{-1} BAP,0.1 mg l^{-1} IAA with different levels of selection.

	total number of explants	explants showing regeneration after 2 weeks	Explants with GUS positive shoots	explants with regeneration after 4 weeks
Experiment 1				
G418 selection level(mg l^{-1})				
10	60	12	0	0
15	60	0	-	0
20	60	0	-	0
Experiment 2				
Kanamycin selection level(mg l^{-1})				
50	60	0	-	0
75	60	0	-	0
100	60	0	-	0

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Chapter 3

Regeneration and transformation of common bean (*Phaseolus vulgaris* L.) using primary leaf explants

Abstract

Common bean is the most important grain legume for direct human consumption and serve as main source of dietary protein for millions of people in developing world. Genetic transformation methods are needed to complement traditional plant breeding methods for common bean improvement. Low transformation frequencies along with other drawbacks make biolistic methods unsuitable for routine transformation of common bean. In an attempt to transform common bean using *Agrobacterium tumefaciens*, primary leaves were used as starting explants. Seeds of common bean great northern cultivar Coyne were germinated for 5 days on MSB5 media with 2% sucrose, pH 5.6, 1mg l^{-1} BAP. Primary leaf explants were prepared from germinated seedlings and inoculated with *Agrobacterium tumefaciens* strain EHA 101. Explants were cultured on MSB5 media amended with 3% sucrose, 10 mg l^{-1} G418 with either 4.68 mg l^{-1} NAA, 2.15mg l^{-1} Kinetin (CM) or 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA (SIM13) after 3 days of co-cultivation for 4 weeks. Fully transformed roots were recovered from primary leaf explants after 4 weeks of culture on CM with an average transformation frequency of 7.5%. Loose white callus also developed from leaf explants, which showed strong GUS expression after transferring the primary leaf explants to MSB5 media with 5 mg l^{-1} Kinetin and 0.15 mg l^{-1} 2, 4-D with 10 mg l^{-1} G418 (SIM). In an attempt to regenerate shoots from primary leaf explants, different factors affecting growth of leaf

explants in culture media were tested. Four different predefined major and minor salt compositions were tested for their effect on leaf explant regeneration. Leaf explants showed significant differences in growth on these 4 media, suggesting the possible role of total nitrogen and ratio of NH_4^+ : NO_3^- in the observed response from the leaf explants.

Keywords: Common bean; Agrobacterium tumefaciens; primary leaf; transformation; regeneration

Abbreviations: BAP- 6-benzyl-aminopurine; NAA – naphthalene acetic acid; 2,4-D – 2,4-Dichlorophenoxyacetic acid; MS – Murashige and Skoog (1962) basal media; Q&L – Quoirin and Lepoivre (1972) basal media; B5 – Gamborg's basal media; MSB5 – media with Murashige & Skoog major and minor salts and Gamborg's B5 vitamins

Introduction

Common bean is the most important grain legume for direct human consumption and are major staple in Eastern and Southern Africa (Broughton et al., 2003). Beans are an important source of dietary protein (22% of the seed weight) and complements cereals for over half a billion people in Latin America (Gepts, 2001; Graham & Ranalli, 1997). The crop is very sensitive to various biotic and abiotic stresses that largely reduce its yield. Common bean yields range from less than 500 kg ha^{-1} in parts of Latin America and Africa to as much as 5000kg ha^{-1} under experimental trails (Graham & Ranalli, 1997).

Different genes conferring resistance to different diseases and insect pest as well as to abiotic stresses are present in alien germplasm (Broughton et al. 2003). However, imperfect chromosome pairing, male sterile F1 hybrids, difficulties in exploiting amphidiploids and requirement of embryo rescue methods hinders the transfer of these useful genes to common bean. (Veltcheva et al. 2005). Genetic transformation can be a powerful and precise tool complementing traditional breeding programs for crop improvement by overcoming these limitations and provides a source to genes from beyond the gene pool accessible only through conventional hybridization (Mukeshimana et al. 2013; Shotwell & Larkins 1991; Raikhel & Last 1993). Efforts to develop efficient transformation protocol for common bean are going on for a long time all around the world, but met with only limited success.

To date, stable common bean transformation has been achieved using direct gene transfer methods mainly particle bombardment or electroporation, but with very low transformation frequencies 0.03% (Russell et al. 1993), 0.9% (Aragão et al. 1996), 0.8 % (Vianna et al. 2004) and 0.66% (Bonfim et al. 2007).

On the other hand common bean transformation using *Agrobacterium* mediated gene transfer has not been successful. Genga *et al*. (1990) transformed cotyledonary node and primary leaf explants with different *Agrobacterium* strains and were able to produce callus on kanamycin selection media but failed to get full explants. McClean *et al*. (1991) transformed cotyledons and hypocotyls using *A.tumefaciens* strain C58Z707/pGA482 and *A.rhizogenes* strain A4RS respectively. They obtained callus and roots but failed to regenerate shoots. Franklin *et al*. (1993) produced transformed GUS positive callus from inoculation of kidney bean with *A.tumefaciens* strain EHA 101. Lewis and Bliss (1994) transformed meristematic regions of different shoot types using stab inoculation with C58 strain, but failed to regenerate any shoots from transformed cells. Kapila et al., (1997) transformed common bean intact leaves through vacuum infiltration of *Agrobacterium* for transient gene expression studies and found high GUS expression of leaf explants with 20-90% of leaf area showing GUS expression. Mukeshimana et al. (2013) transformed embryo axes of common bean cultivar “Merlot”, but were unable to produce whole plants. Collado et al. (2015) reported stable transformation of common bean using *A.tumefaciens* strain EHA 105 through indirect organogenesis. Transformation frequency reported was 2.8%. This protocol seems effective, but its repeatability and effectiveness has to be evaluated in coming future.

Several reports are available for whole plant regeneration in common bean using shoot apex cultures (Kartha et al. 1981; Martins & Sondahl, 1984), cotyledonary node and primary leaf nodes (Mohamed et al. 1992), cotyledonary node devoid of axillary buds (McClean & Grafton, 1989; Mariotti *et al.* 1989), cotyledonary node with axillary buds (Franklin et al. 1991), embryo derived callus (Zambre et al. 1998). However, no follow up reports of *Agrobacterium tumefaciens* mediated transformation using these regeneration protocols is available till date.

Juvenile leaves were reported to be highly responsive to *Agrobacterium* infections in common bean (Genga et al., 1989; Kapila et al. 1997; Mukeshimana et al., 2013). However, no reports for detailed study on use of primary leaves as explants for common bean transformation are available. Leaf explants were used successfully to generate transformed plants in other legumes like *Cajanus cajan* (Dayal et al. 2003), *Medicago truncatula* (Araújo et al. 2004; Trinh et al., 1998), *Vigna radiata* (Mahalakshmi et al., 2006), *Medicago sativa* (Austin et al., 1995; Shao et al., 2000), using *Agrobacterium tumefaciens* mediated gene transfer, indicating the potential of this explant for legume transformation. So the objective of this study was to (i) investigate the potential of primary leaves of common bean great northern cultivar Coyne as an explant for *Agrobacterium tumefaciens* mediated transformation (ii) to investigate factors affecting regeneration of leaf explants.

Materials and Methods

Plant material

Seeds of *P. vulgaris* L. great northern cultivar "Coyne" (Urrea et al. 2009) were provided by Dr. Carlos Urrea, Associate Professor, Agronomy and Horticulture University of Nebraska-Lincoln. Seeds were surface sterilized by an overnight exposure to chlorine gas (Di et al. 1996). Sterilized seeds were germinated, in 100 X 20 mm Petri dishes, on germination media (GM) containing MS inorganic salts (Murashige & Skoog, 1962) with Gamborg's B5 vitamins (Gamborg et al. 1968) (denoted as MSB5), 2%sucrose, 0.8% agar and 1 mg l^{-1} BAP. pH was adjusted to 5.6 before autoclaving for 21 minutes at 121^0C and 1.07 kg cm^{-2}. The plates were stacked 5 high and placed in plastic bags in which 4, approx. 3 inch, slits were made with scissors. Seeds were germinated for 5 days in a growth room, at 24^0 C, 18/6 light regime, under a light intensity of approximately 150 μmol s^{-1} m^{-2}.

Explant preparation

Primary leaf explants were prepared from 5 day old seedlings after removing cotyledons. Leaf explants of 5 X 5 mm were cut with sterile scalpel (with No. 11 scalpel blade) excluding outer leaf margin and midrib.

Agrobacterium strain and culturing conditions

Agrobacterium tumefaciens strain EHA 101 (Hood et al. 1986) harboring binary plasmid pPTN 1043 was used for inoculation. pPTN 1043 contains the *neomycin phosphotransferase* gene (*npt*II) driven by cauliflower mosaic virus 35S promoter and an intron interrupted *β-glucronidase* gene (*gus*A) which is controlled by 35S promoter (Figure 3.1.a). Strain EHA 101 with binary plasmid pPTN 289 which contains *bar* gene as selection marker driven by *nopaline synthase(nos)* gene promoter and intron interrupted *gus*A gene as reporter gene driven by 35S promoter was used for control treatments (Figure 3.1. b). Single colonies of each strain were streaked on plates with 25ml of Luria Bertani (LB) media containing 25mg l^{-1} of each kanamycin, sterptinomycin, spectinomycin and chloramphinol antibiotics and incubated in 28^0C for 2 days. Day before inoculation *Agrobacterium* cultures from these plates were grown in 5 ml YEP medium (10 g l^{-1} peptone, 5 g l^{-1} yeast extract and 5 g l^1 NaCl, pH 7.0) amended with appropriate antibiotics to an OD_{650} = 0.5 to 1.0 at 28^0C. Bacterial cultures were centrifuged at 3500 rpm for 10 minutes and the pellets re-suspended in to final OD_{650} = 0.5 to 1.0 in liquid co-culture media containing 10^{-1} MS inorganic salts (Murashige & Skoog, 1962) with 10^{-1} Gamborg's B5 vitamins, 3% sucrose, 200 μM acetosyringone (AS), with either 4.68 mg l^{-1} NAA, 2.15 mg l^{-1} Kinetin or 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA, pH 5.4 and buffered with 20mM MES. All growth regulators, vitamin components and AS were filter sterilized and added post autoclaving.

Plant transformation

Leaf explants were immersed in *Agrobacterium tumefaciens* EHA 101(pPTN 1043) inoculum for 30 minutes and then co-cultured on 100 X 15 mm petri plates containing co-culture media solidified with 0.5% agar. For control experiments, leaf explants were inoculated with pPTN 289. Co-cultivation plates were overlaid with a piece of Whatman #1 filter paper. Leaf explants (6 explants per plate) were cultured abaxial side down on the co-cultivation plates and wrapped with parafilm. Explants were co-cultivated for 3 days at 24^0 C, 18/6 light regime, under a light intensity of approximately 80 μmol $s^{-1}m^{-2}$.

Following the co-cultivation period explants were briefly washed in MSB5 media supplemented with 4.68 mg l^{-1} NAA, 2.15 mg l^{-1} Kinetin, 3% sucrose, 75 mg l^{-1} of each ticarcillin, cefotaxime and vancomycin, buffered with 3mM MES and pH adjusted to 5.7 along with either 4.68 mg l^{-1} NAA and 2.15 mg l^{-1} kinetin or 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA. Growth regulators, vitamins and antibiotics were filter sterilized and added to media post autoclaving. After washing, explants were cultured in 100 X 20 mm petri plates, by placing abaxial side in touch with medium, containing 50 ml of MSB5 media supplemented with 3% sucrose, 50 mg l^{-1} of each ticarcillin, cefotaxime and vancomycin, solidified with 0.8% purified agar amended with 10 mg l^{-1} G418 along with either 4.68 mg l^{-1} NAA and 2.15 mg l^{-1} kinetin (media referred as Callus Media CM) or 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA (media abbreviated as SIM13). Plates were wrapped with 3M pressure sensitive tape and cultured at 24^0 C, 18/6 light regime, under a light intensity of approximately 150 μmol $s^{-1}m^{-2}$.

After 2 weeks of culture, leaf explants showing nodal growth on respective media were recorded and Histochemical GUS assay was performed. Then leaf explants showing

nodal growth were transferred to Shoot Induction Media (SIM) which was MSB5 media with 5 mg l^{-1} Kinetin, 0.05 mg l^{-1} 2,4-D, 3% sucrose, 50 mg l^{-1} of each ticarcillin, cefotaxime and vancomycin, solidified with 0.8% purified agar and amended with 10 mg l^{-1} G418. After 4 week culture on SIM explants were transferred to MSB5 media with 3% sucrose, 50 mg l^{-1} of each ticarcillin, cefotaxime and vancomycin, 0.8% agar, amended with 2 mg l^{-1} BAP and 10 mg l^{-1} G418.

For root growth, after 2 weeks culture on CM and SIM13 media, explants showing nodal growth were transferred to fresh CM and SIM13 media respectively, with 10 mg l^{-1} G418. Following an additional 2 weeks of culture number of explants showing root growth, number of roots and length of roots were recorded on respective media.

Histochemical GUS assay

Histochemical GUS assays (Jefferson et al. 1987) were conducted as described by Zhang et al. (1999). Leaf tissue was submerged in a substrate solution composed of 0.1 M $NaHPO_4$ buffer (pH 7.0), 0.5 mM K_3 [Fe $(CN)_6$], 0.5mM K_4 [Fe $(CN)_6$, 10mM EDTA and 800 mg l^{-1} X-Gluc. Methanol and Triton-X100 were subsequently added to final concentrations of 20% and 0.06%, respectively. Common bean tissue was allowed to incubate in the X-Gluc substrate for 5-8 hours at 37^0C. The tissue was subsequently cleared in 70% ethanol prior to visualization.

Fifteen leaf explants from each replication (total 30 leaf explants per treatment) were chosen randomly after 3 days of co-cultivation of the explants and were washed in wash media to remove excess *Agrobacterium* sticking on explant surface. Total thirty explants

were used for histochemical GUS assay from each treatment. Explants showing blue color (at least one blue spot) were considered as positive. After 2 week of culture CM, fifteen explants from each replication were used for histochemical GUS assay (total thirty explants). From SIM13 all 7 explants were analyzed for GUS expression. Explants exhibiting at least one blue spot in new growth were considered positive.

For callus growing on SIM, half of each callus was used for GUS expression analysis after two and four weeks of culture. Localized regions of transformed cells, as indicated by a blue precipitate were detected in all studies.

For root histochemical assay, explants showing root development were immersed in X-Gluc along with roots. Explants with at least one root showing blue precipitate throughout the root were counted.

Major and minor nutrients comparison

Four different basal salt mixtures, MS basal salt mixture (Murashige & Skoog, 1962), Quoirin and Lepoivre basal salt mixture (Quoirin & Lepoivre, 1977) abbreviated as QL, Lloyd and McCown's woody plant basal salt mix (McCown & Lloyd, 1981) abbreviated as MC, and Hoagland's No. 2 basal salt mixture (Hoagland & Arnon, 1950) were tested for their effect on growth of leaf explants. These four different basal salt mixtures differ in type and concentration of nutrients especially for inorganic nitrogen (Table 1). Four different media were prepared with 3% sucrose, 3mM MES, B5 vitamins, 1 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and solidified with 0.8% agar and by adding 4.3 g l^{-1} MS, 3.6 g l^{-1} QL, 2.3 g l^{-1} MC or 1.6 g l^{-1} Hoagland's basal salt mix were added to make four media types.

MS, MC and Hoagland's basal salt mix were purchased from Sigma-Aldrich while Q&L basal salt mix was purchased from Phyto technology laboratories KS USA (lot no. 15K0673023A). pH of all media were set to 5.7 before autoclaving for 21minutes. Filter sterilized vitamins and growth regulators were added to media post autoclaving. Primary leaf explants were prepared from five day old seedlings. Six explants per petri plate were placed abaxial side touching the media in 100 X 50 mm petri plates. Explants were cultured in 18/6 light/dark regime at 24^0C. After two weeks of culture, explants were analyzed for nodal growth and were sub-cultured to fresh respective media.

Statistical procedures

Experiments were conducted as a randomized design with 3 replications per treatment. Each replication was represented by forty leaf explants (total 120 leaf explants per treatment). For basal salt comparisons, each replication was represented by thirty leaf explants (total 90 leaf explants) per treatment. Means were compared using PROC GLM (SAS version 9.4). Analysis of variance (ANOVA) was used to test statistical significance.

Results and Discussion

Susceptibility of leaf explants for Agrobacterium infection

To check the susceptibility of primary leaf explants for *Agrobacterium* infection, 10 explants from each replication (30 explants from each treatment) were taken and histochemical GUS assay was done. Out of 30 explants tested for GUS expression all the explants showed dark blue color mainly at the margins of leaf explants (Table 3.1). Along with leaf margins, explants showed varying degree of area covered by blue precipitate. Blue color appearance varied from on the margins to whole explant (Figure 3.2). High GUS expression on the explant margins was due to wounding of the explants that occurred during explant preparation. These results are in agreement with previous findings (Genga et al. 1989; Kapila et al. 1997; Mukeshimana et al. 2013), where they reported high GUS expression of leaf explants inoculated with *Agrobacterium tumefaciens.* High level of GUS expression indicates that in leaf explants number of cells that contained and express the introduced gene were high. Higher number of transformed cells will increase the chances of getting high transformation efficiencies, which is the main limiting factor of common bean transformation procedures.

Callus/nodal growth

Leaf explants showed nodal growth after 2 weeks culture on both media. On CM significantly higher number of explants 56 out of total 120 explants showed nodal growth compared to number of explants on SIM 13, where only 7 out of 120 explants showed nodal growth (Figure 3.3. a, b). Average number of explants on CM with nodal growth

was significantly higher than the average number of explants with nodal growth on SIM13 (Table 3.2). Higher concentration of growth hormones in CM led to increased number of explants with nodal growth compared to SIM13. Thirty total explants (10 from each replication) from CM and all 7 explants from SIM13 were analyzed for GUS expression. Twenty nine out of 30 explants from CM and all the 7 explants from SIM13 exhibited strong blue precipitate in the nodal growth (Figure 3.3. (c)). This strong GUS expression indicates the stable integration of transgene. On CM on average 45% of explants produced nodal growth , which is significantly higher than 5.83% explants on SIM13 (Table 3.2). Remaining 26 explants were transferred to SIM. After 4 weeks culture on SIM, 22 out of 26 explants showed growth of white to light brown callus (Figure 3.4. a). Despite of higher concentration of kinetin in the media, presence of 2, 4-D might resulted in production of loose callus, as 2, 4-D promotes callus production (Sen et al. 2014). When these callus producing explants were analyzed for GUS assay, 20 explants exhibited strong blue coloration in the new callus growth (Figure 3.4. b), which showed that the new callus growth was originated from transformed cells. When this callus was transferred to MSB5 media with 3% sucrose, 2mg/l BAP and 10mg/l G418 selection, callus turned brown and died within 2 weeks.

Root development

When leaf explants were cultured on CM and SIM13 for 4 weeks, 20% of leaf explants (23 out of 120) developed roots (Figure 3.5. a), while on SIM13 only 1 explant showed growth of single root (Table 3.3). Higher number of explants producing roots on CM was

probably due to higher amount of NAA (4.68 mg l^{-1}) compared to kinetin (2.15 mg l^{-1}) in the media. Higher auxin to cytokinin ratio promotes root growth while higher cytokinin to auxin ratio suppress root growth and increase shoot growth. (Smigoki & Owens., 1989). This fact explains the production of only one root on SIM13. Roots were developed from the new growth on the leaf explants, indicating the organogenic potential of leaf explants. When the explants showing root growth were analyzed for GUS expression, nine explants out of 23 on CM had fully transformed roots (Figure 3.5. b, c), as these roots exhibited strong GUS expression throughout the root. Single root developed on SIM13 was also fully transformed. Production of fully transformed roots further support the fact that leaf explants can yield stable transformation events and can produce transformed plants. Leaf explants generated fully transformed roots with a frequency of 7.5% (Table 3.3) when cultured on CM.

As indicated by high initial GUS expression and then continuous GUS expression of callus growing on SIM and GUS expression in whole roots suggest that leaf explants are highly susceptible to *Agrobacterium* infections and has organogenic potential. All these information suggest that leaf explants might lead to production of transgenic common bean through *Agrobacterium* mediated transformation to produce transgenic common beans.

Major and minor salts comparison

Four different predefined compositions of major and minor nutrients were tested for their effect on regeneration of primary leaf explant of common bean. These were selected

based on nitrogen component and NH_4^+:NO_3^- ratio. Murashige and Skoog (MS) basal salts has high concentration of total nitrogen with high ammonium (20.62 mmol l^{-1}) and nitrate (39.41 mmol l^{-1}) compared to Quoirin and Lepoivre basal salt mixture(QL) that has 4.99 mmol l^{-1} NH_4^+ and 22.88 mmol l^{-1} NO_3^-, Hoagland's No.2 basal salt mix(HO) has 2.31 mmol l^{-1} NH_4^+ and 10mmol/l NO_3^-, with McCown's woody plant basal salt mix (MC) that has 4.99 mmol l^{-1} NH_4^+ and 7.34mmol/l NO_3^-. After 2 weeks culture on these media leaf explants showed different response to all these nutrient mixes. On MS and MC media significantly higher number of explants showed nodal growth compared to QL. No explant showed any nodal growth on Hoagland's media (Table 3.4). Significant differences also observed in blackening of the nodal growth. Explants on MC and QL showed significantly more blackening of nodal growth compared to Hoagland's and MS media, with explants on MS media showing least amount of blackening of nodal growth (Table 3.5) (Figure 3.6). Blackening of media and nodal growth was possibly due to the production of phenolics. Reports are available on the effect of nitrogen in culture media and production of phenolics. Reduction in nitrogen in culture media increases the production of phenolics and other secondary metabolites (Wojtania et al. 2015; Shohael et al 2013). Lower nitrogen in MC and QL compared to MS might be the possible reason for increased blackening of nodal growth. Further subculture of explants onto respective media was done and after another 2 weeks, explants on MS media produced large brown loose callus (Figure 3.7. a). This might be the effect of continuous exposure to high ammonium present in MS. There might be possibility that other components except nitrogen might be responsible for that loose brown callus. On QL and MC media, no

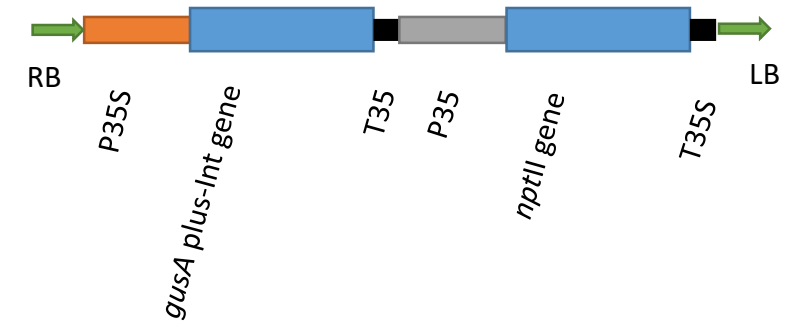
loose callus was formed (Figure 3.7. b, c). On QL media, nodal growth was covered by dense blackening. On MC media, explants turned white and watery. While on Hoagland's media, explants did not show any growth even after 4 weeks of culture (Figure 3.7. d).

Varying response of leaf explants to major and minor salts suggest that major and minor salts and especially nitrogen, has an important role in leaf explant regeneration in common bean great northern cultivar Coyne. As limited work has been done on leaf explant regeneration in common bean (Malik and Saxena 1991) compared to other explants like cotyledonary node, embryonic axes, apical meristems where several reports show recovery of full plants from these explants (Mohamed et al. 1992; McClean and Grafton 1989; Franklin et al. 1991). Hence, more investigation is needed for optimizing different factors that might affect regeneration of leaf explants in common bean.

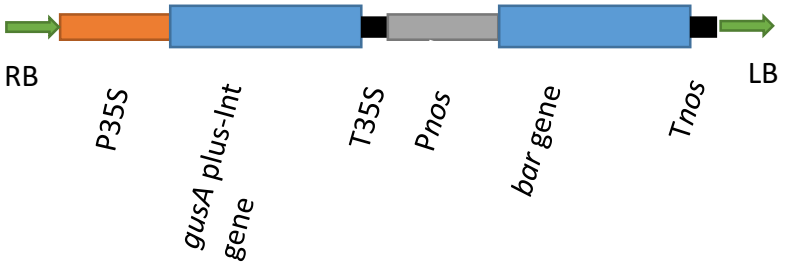
Future Directions

Results from these experiments suggest that leaf explants has a potential to serve as explant for common bean transformation. However, an efficient regeneration method from primary leaf explants is necessary. Results of our preliminary studies on regeneration of leaf explants suggest that in the future, more elaborative work is needed to analyze different factors that affect regeneration of leaf explants leading to optimization of regeneration method for primary leaf explants of common bean.

Figures

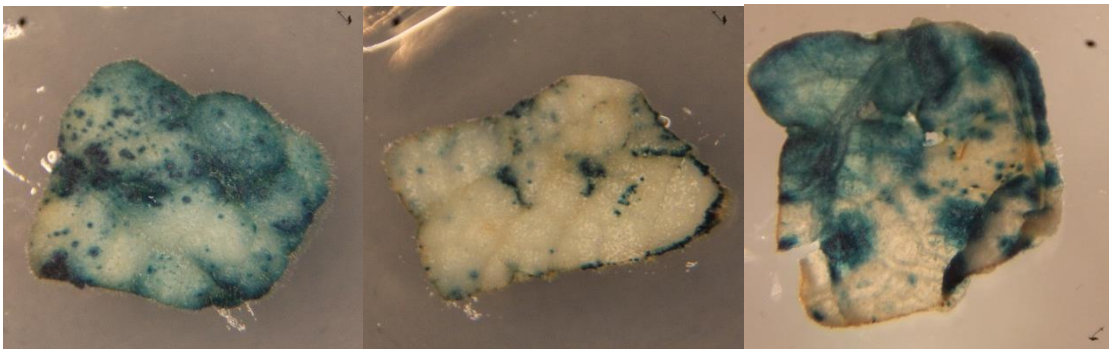


(a)

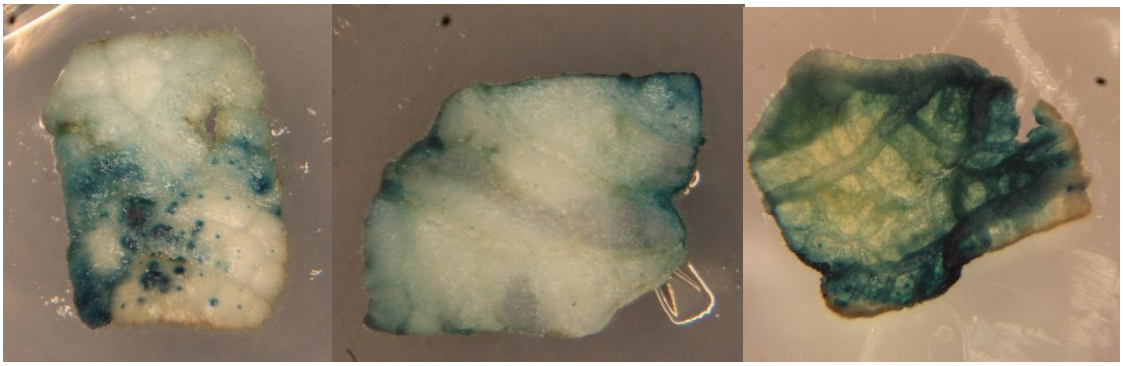


(b)

Figure 3.1. Schematic diagram of the T-DNA region from the binary vectors (a) pPTN 1043 and (b) pPTN 289. RB and LB, T-DNA right and left border sequences respectively; P35S, Cauliflower mosaic virus 35s promoter; *gusA* plus-int, β-*glucuronidase* gene interrupted by intron; T35S, cauliflower mosaic virus 35s polyA tail; *npt* II , *neomycin phosphotransferase* II conferring G418 resistance; P*nos* and T*nos*, *nopaline synthase* gene promoter and terminator respectively; *bar*, bialaphos resistance gene

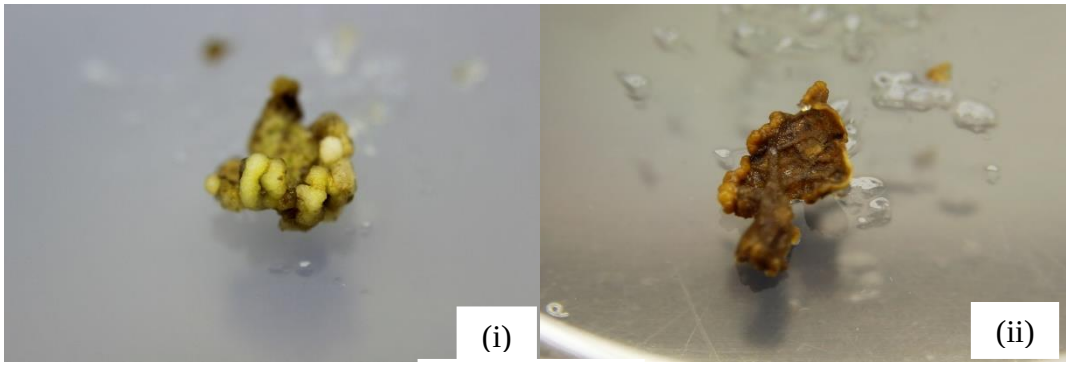


(a)

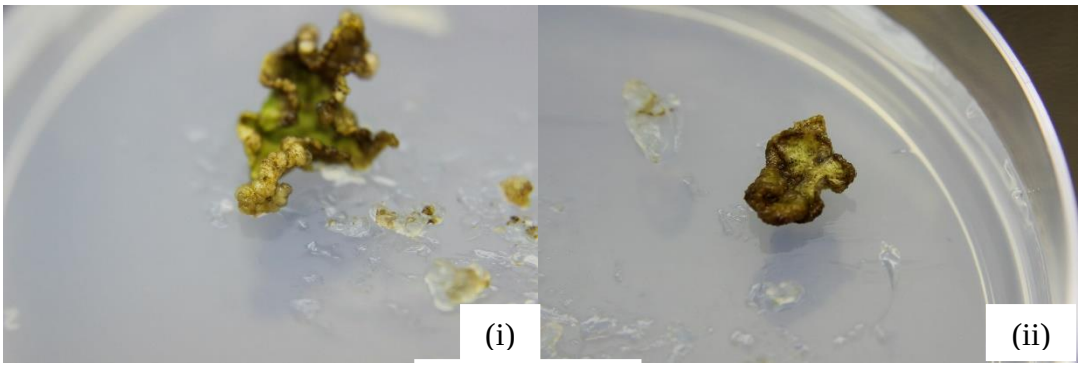


(b)

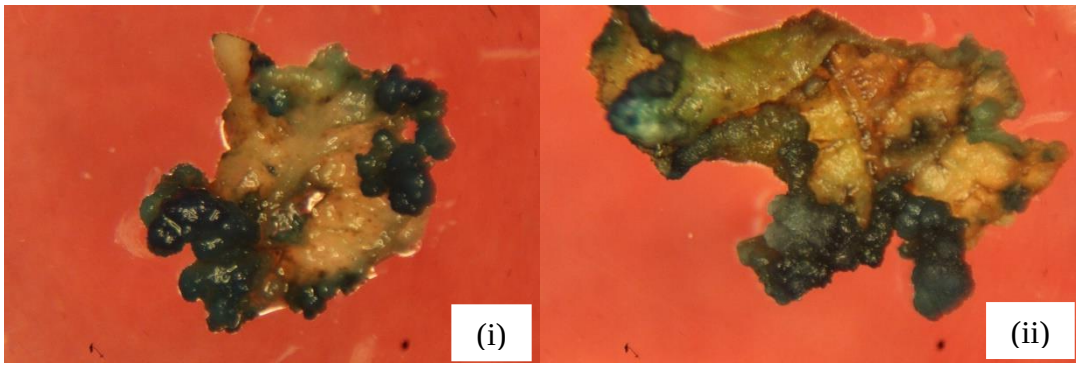
Figure 3.2. Histochemical GUS assay of primary leaf explants of ‘Coyne’ after 3 days of co-cultivation (a) primary leaf explants co-cultivated on CM co-culture media (b) primary leaf explants co-cultivated on SIM 13 co-culture media



(a)

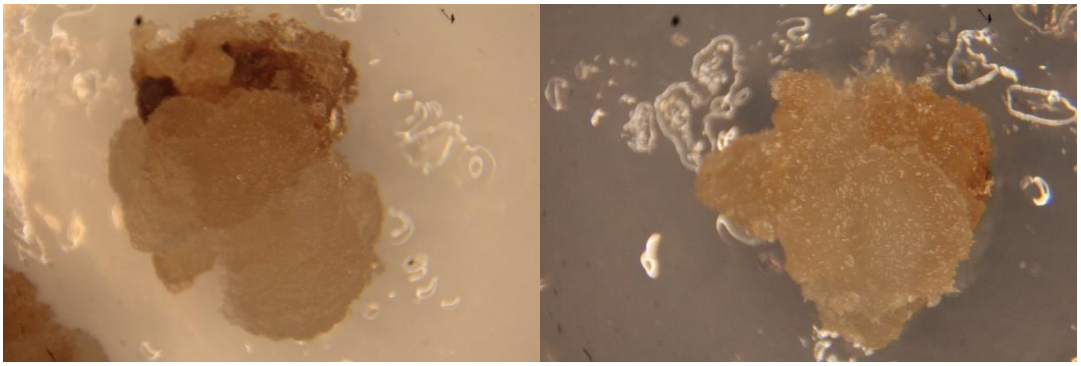


(b)

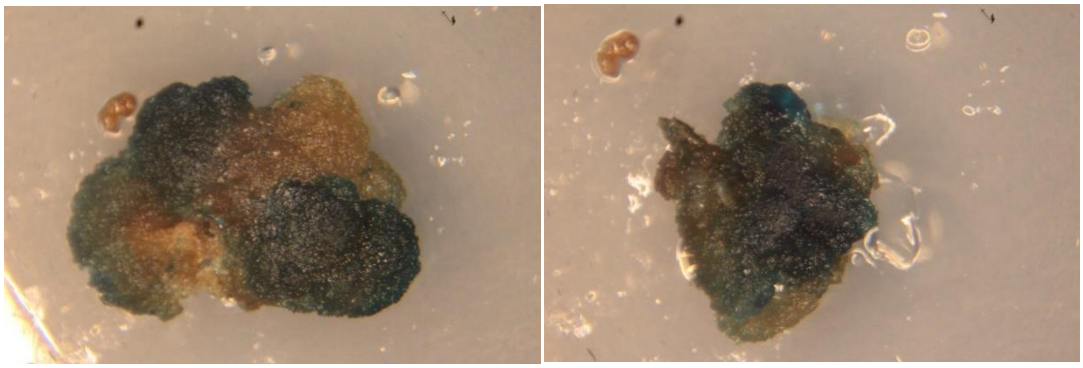


(c)

Figure 3.3. Growth of primary leaf explants of 'Coyne' after 2 weeks culture on 10mg l^{-1} G418 selection (a) (i) explant growth on CM media (a) (ii) control explant growth on CM media (b) (i) explant growth on SIM 13 media (b) (ii) control explant on SIM 13 media (c) histochemical GUS assay (i) explant cultured on CM (ii) explant cultured on SIM 13

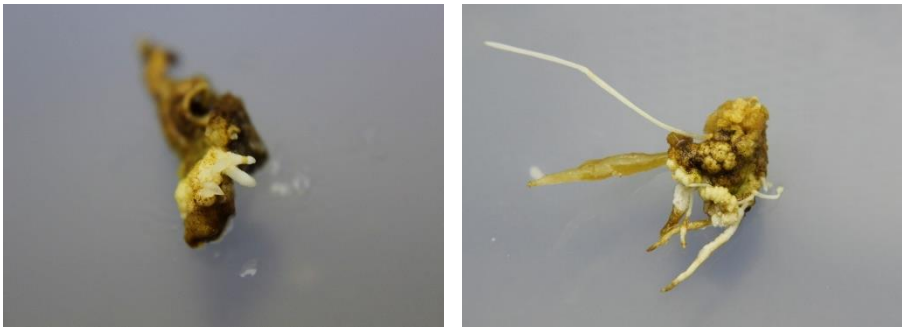


(a)

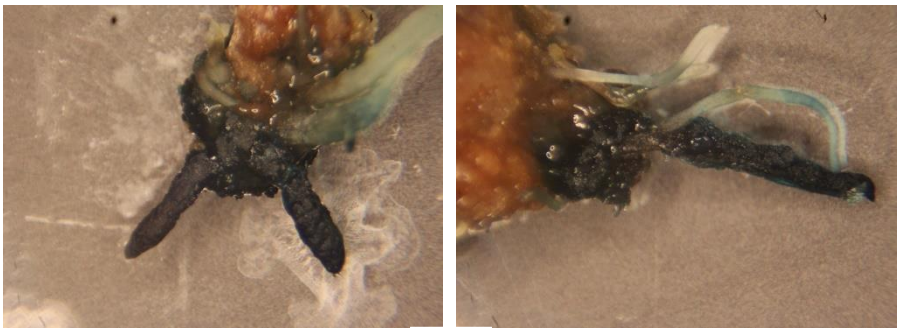


(b)

Figure 3.4. Callus growth from leaf explants of ‘Coyne’ after 2 weeks of culture on SIM (a) callus growth (b) histochemical GUS assay of callus showing GUS expression in new growth



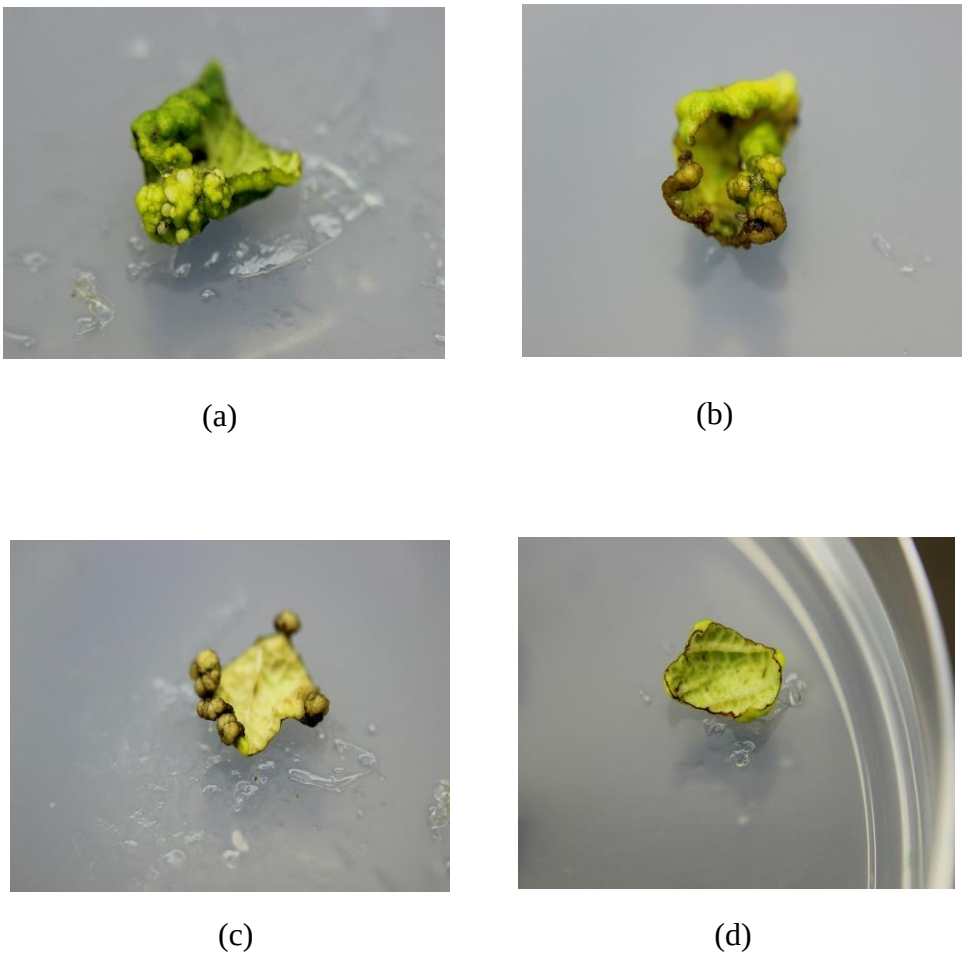
(a)



(b)

(c)

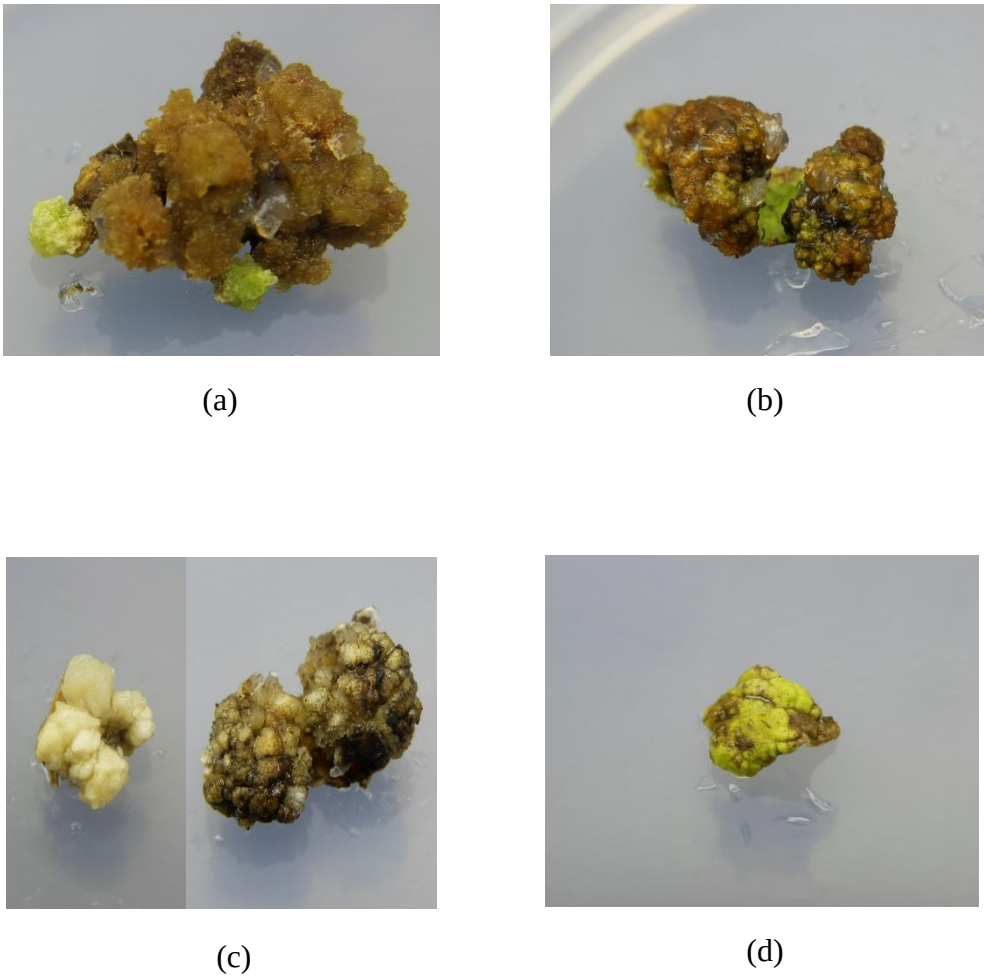
Figure 3.5. (a) root growth on leaf explants of ‘Coyne’ after 4 weeks culture on CM (b) histological GUS assay of explants showing root growth (c) GUS positive roots from leaf explants (upper row) along with chimeric roots (bottom two roots)



(a) (b)

(c) (d)

Figure 3.6. Growth of leaf explants of ‘Coyne’ after 2 weeks culture on media with 3% sucrose, B5 vitamins, 3mM MES, 1mg l^{-1} BAP, 0.5mg l^{-1} NAA with different major and minor salts (a) MS basal salts (b) Q&L basal salts (c) McCown’s woody plant basal salts (d) Hoagland’s No.2 basal salts



(a) (b)

(c) (d)

Figure 3.7. Growth of leaf explants of ‘Coyne’ after 4 weeks culture on media with 3% sucrose, B5 vitamins, 3mM MES, 1mg l^{-1} BAP, 0.5mg l^{-1} NAA with different major and minor salts (a) MS basal salts (b) Q&L basal salts (c) McCown’s woody plant basal salts (d) Hoagland’s No.2 basal salts

Tables

Table 3.1. Response of primary leaf explants to *Agrobacterium* infection as shown by histochemical GUS assay after 3 days of co-cultivation

Media	Replication	Total Explants Tested For Gus	Gus Positive Explants	Transformation Efficiency (%)[a]
Callus	1	10	10	100
	2	10	10	100
	3	10	10	100
	TOTAL	30	30	100
Sim13	1	10	10	100
	2	10	10	100
	3	10	10	100
	TOTAL	30	30	100

[a]Transformation efficiency(%) = $\left(\frac{\text{number of explants with GUS positive roots}}{\text{total number of explants}}\right)$ X 100

Table 3.2. Nodal growth response of primary leaf explants of great northern cultivar "Coyne" after 2 weeks of culture

Media	replicate	total number of explants	Number of explants with callus growth	Number of explants with GUS +ve callus (number of explants tested)	GUS positive explants (%)[a]	Number of explants transferred to SIM	Number of explants with callus growth	Number of explants with GUS positive callus
CM	1	40	21	9(10)	47.25	11	9	9
	2	40	17	10(10)	42.5	7	7	7
	3	40	18	10(10)	45	8	6	6
						26	22	22
	total	120	56	29(30)				
	mean±SE		18.6±1.02 a		45±1.44 a			
SIM 13	1	40	3	3(3)	7.5			
	2	40	2	2(2)	5			
	3	40	2	2(2)	5			
	total	120	7	7(7)				
	Mean ± SE		2.33 ± 0.33 b		5.83 ± 0.83 b			

[a] GUS positive (%) = $\left(\frac{\text{number of explants with GUS positive callus}}{\text{number of explants tested for GUS}}\right)$ X $\left(\frac{\text{number of explants with callus growth}}{\text{total number of explants}}\right)$ X 100

Means with same letter are not significantly different ($\alpha = 0.05$)

Table 3.3. Transgenic root development from primary leaf explants of common bean great northern cultivar “Coyne” after 4 weeks of culture CM and SIM media

Media	Replicate	Total number of explants	Number of explants with callus growth after 2 week culture	Number of explants with root growth	Number of explants with GUS +ve roots	Transformation efficiency (%)[a]
callus	1	40	15	7	2	5
	2	40	19	8	4	10
	3	40	17	8	3	7.5
	total	120	51	23	9	7.5
	Mean ± SE		17 ± 1.15 a	7.67 ± 0.33 a	3 ± 0.577 a	
SIM13	1	40	2	0	0	0
	2	40	4	1	1	2.5
	3	40	3	0	0	0
	total	120	9	1	1	0.83
	Mean ± SE		3 ± 0.577 b	0.33 ± 0.33 b	0.33 ± 0.33 b	

[a] Transformation efficiency = $\left(\frac{\text{number of explants with GUS positive roots}}{\text{total number of explants}}\right)$ X 100

Means with same letter are not significantly different ($\alpha = 0.05$)

Table 3.4. Effect of different major and minor salts on nodal growth of primary leaf explants after 2 weeks of culture

	Number of explants with nodal growth			
Replication[1]	Murashige and Skoog (MS)	Quoirin and Lepoivre (QL)	McCown's (MC)	Hoagland's No.2 (HO)
1	28	6	24	0
2	11	9	24	0
3	21	10	11	0
Mean ± SD	20 ± 8.54 a	8.33 ± 2.08 b	19.667 ± 7.5 a	0 ± 0 b

[1] 30 primary leaf explants on each replication (6 explants per petri plate and 5 plates representing each replication)

Means followed by the same letter are not significantly different ($\alpha = 0.05$)

Table 3.5. Effect of different major and minor salts compositions on average blackening score of primary leaf explants after 2 weeks of culture

Replication[1]	Murashige and Skoog (MS)	Quoirin & Lepoivre (QL)	McCown's (MC)	Hoagland's No.2(HO)
1	0.164	1.2	1.2	0.532
2	0.1	1.32	2.2	0.464
3	0.232	1.26	1.6	0.364
Mean ± SD	0.1653 ± 0.066 b	1.26 ± 0.060 a	1.68 ± 0.50 a	0.45 ± 0.084 b

[1] 30 primary leaf explants on each replication (6 explants per petri plate and 5 plates representing one replication)

Means followed by the same letter are not significantly different ($\alpha = 0.05$)

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