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Development of a highly efficient, repetitive system of organogenesis in soybean (*Glycine max* (L.) Merr).

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Abstract A highly efficient, repetitive system of organogenesis was developed in soybean. Seeds of soybean cv. 'White hilum' pretreated with TDZ formed multiple bud tissue(s) (MBT) at the cotyledonary nodes. MBT initiation occurred only if the axillary buds were not removed from the cotyledonary node. The best MBT formation was achieved by pretreating the seeds for 1 week on medium supplemented with 0.1 mg/l TDZ, followed by culture of the cotyledonary node on medium supplemented with 0.5 mg/l BA for 4 weeks. Culture of the MBT on medium supplemented with 0.1 mg/l TDZ resulted in the proliferation of MBT. MBT was maintained in this way for 12 months. Three hundred thirty six shoots were obtained when 1 g of MBT was subcultured on medium supplemented with 0.5 mg/l BA. Plants were rooted on medium without growth regulators. The regenerated plants grew normally in the greenhouse. Unfortunately, they did not set seeds because of the long-day conditions during growth. This system was successfully applied in three other genotypes.

Keywords Soybean (*Glycine max* (L.) Merr) · Cotyledonary node · Regeneration · Thidiazuron · TDZ

Introduction

Soybean (*Glycine max* (L.) Merr.) is grown on more areas worldwide than any other dicotyledonous crop. Commercial breeding is still very important for the genetic improvement of the crop. However, breeding is difficult due to the fact that soybean is a self-pollinating crop and the genetic variation among soybean varieties is narrow. Genetic modification has been used for producing varieties with traits that are not available in the soybean gene pool. The regeneration system used to produce genetically modified plants was somatic embryogenesis from immature seeds (Christou et al. 1989) or organogenesis from cotyledonary nodes (Finer and McMullen 1991).

Somatic embryogenesis is an efficient system to produce genetic modified plants, however, it is genotype specific and accompanied with a high level of somaclonal variation in the regenerated plants (Finer and Nagasawa 1988; Parrot et al. 1989; Finer and McMullen 1991). Organogenesis is less genotype dependent and has become routine in several laboratories (Wright et al. 1986, 1987a, 1987b; Barwale et al. 1986; Dan and Reighceri 1998). The regeneration is based on proliferation of meristems in the cotyledonary node. However, recovery of transgenic plants capable of transmitting the target genes to R1 is very low (Christou et al. 1990). Olhoft et al. (2001) stated that the efficiency of soybean transformation has to be improved 5–10 times before one person can produce 300 transgenic lines per year. The efficiency of the organogenic system for genetic modification could be improved if the number of shoots per explant is increased or if the number of meristematic cells in explants is increased.

In pea, where genetic modification is also based on organogenesis from cotyledonary nodes, a highly efficient regeneration system was obtained by subculturing nodes on thidiazuron (TDZ) supplemented medium. As a result tissues were formed covered with very small buds. These bud containing tissues were maintained by repetitive subculture (Tzitzikas et al. 2004) and were successfully used in genetic modification. This report describes the establishment of a highly efficient, repetitive organogenic system in soybean based on the use of TDZ.

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Materials and methods

Plant material, seed pretreatment and initiation of multiple bud tissue (MBT)

Soybean seeds (*Glycine max* (L.) Merr. cv White hilum) were sterilized in 70% ethanol for 1 min and then in 0.5% (w/v) sodium hypochlorite for 20 min, followed by four rinses with sterile water. Seeds were cultured in Petri dishes (10 seeds/dish) containing medium supplemented with Murashige and Skoog (1962) salts and vitamins, 3% sucrose, 0.8% micro agar (Duchefa) (MS) and in MS supplemented with 0.1 mg/l TDZ or 0.2 mg/l TDZ or 0.5 mg/l TDZ or 0.5 mg/l BA. Cotyledons, epicotyl and hypocotyl were removed under a stereomicroscope to avoid wounding of the axillary buds after 7 days. Cotyledonary nodes with or without axillary buds were taken as explants and cultured on fresh media. After 3 weeks the explants were transferred to fresh medium.

Three replicates with 10 seeds per replicate were used for each treatment. The experiments were repeated at least three times. All cultures were kept in a growth chamber with a temperature of 25°C, an irradiance of 40 μmol m^{-2} s^{-1} and a photoperiod of 16 h. using cool white fluorescent tubes.

Multiplication of multiple buds tissue

MBT formed on the cotyledonary nodes after 3–6 weeks of culture, was transferred to MS supplemented with 0.5 mg/l BA, or 0.1 mg/l TDZ, or 0.3 mg/l TDZ or 0.5 mg/l TDZ for multiplication. Every 2 weeks the MBT tissue was transferred to fresh medium. In a separate experiment the increase in fresh weight and development of the tissue was recorded. The experiment consisted of three replicates and per replicate 10 pieces of MBT (±60 mg per piece). Also the shoot nodes derived from MBT were subcultured on MS supplemented with 0.1 mg/l TDZ.

Plant regeneration

MBT tissue was subcultured on MS supplemented with 0.5 mg/l BA, or 0.2 mg/l BA, or 0.5 mg/l GA3 for shoot elongation. The experiment consisted of three replicates and per replicate 10 pieces of MBT (±60 mg per piece). Every 3 weeks the tissue was transferred to fresh medium. The number of shoots was counted 6 weeks after culture. Isolated single shoots were transferred to MS medium for rooting. Rooting efficiency (percentage of shoot producing roots) was recorded after 4 weeks of culture. Plantlets were transferred to the greenhouse for acclimatization and growth. The plants were first grown in small pots. After about 1 month of growth they were transferred to 10 l pots.

Results

Effect of seed pretreatment with cytokinin Soybean seeds germinated on all the tested media within 2 days. No obvious differences were observed in germination frequency between the different treatments (data not shown). However, the growth and development of the seedlings differed significantly between the treatments. Seed cultured on MS germinated normally (Fig. 1a). Seeds cultured on media supplemented with cytokinins germinated abnormally: enlarged cotyledons, thick and short hypocotyls, thick and short roots which were swollen at the end with no or only a few, small lateral roots (Fig. 1b). No callus was formed on intact seedlings.

Seven days after germination the axillary buds at the cotyledonary nodes of the seedlings cultured on cytokinin supplemented media expanded in size and became visible with the naked eyes (Fig. 1c). No axillary buds were visible with the naked eyes on the seedlings cultured on MS (Table 1). The axillary buds of seeds cultured on MS 0.5 mg/l BA were bigger than those of seeds cultured on TDZ supplemented media. At this stage the cotyledons, epicotyls and hypocotyls were removed and the remaining cotyledonary nodes were taken as explants and transferred to fresh media for MBTs formation.

Table 1 The effect of seed pretreatment on formation of multiple bud tissue (MBT) and shoots

Medium for seed pretreatment	Medium for MBT induction	Seeds with expansion of axillary buds (%)	Size MBT (mm^2) per seed explant (6th week)	# of shoots per seed (6th week)
MS	MS	0	0^a	0
MS	MS0.5 mg/l BA	100	0^a	2–3
MS 0.1 mg/l TDZ	MS 0.5 mg/l BA	100	21^c	0
MS 0.2 mg/l TDZ	MS 0.5 mg/l BA	100	10^b	0
MS 0.5 mg/l TDZ	MS 0.5mg/l BA	100	12^b	0
MS 0.5 mg/l BA	MS 0.5 mg/l BA	100	12^b	0
MS 0.1 mg/l TDZ	MS 0.5 mg/l TDZ	100	13^b	0
MS 0.1 mg/l TDZ	MS	100	0^a	3–6

Note. 30 explants were used per seed pretreatment (1st week), MBT induction (2nd–6th week). Values with different letters are statistically different ($p<0.05$) with the Fisher's protected LSD test

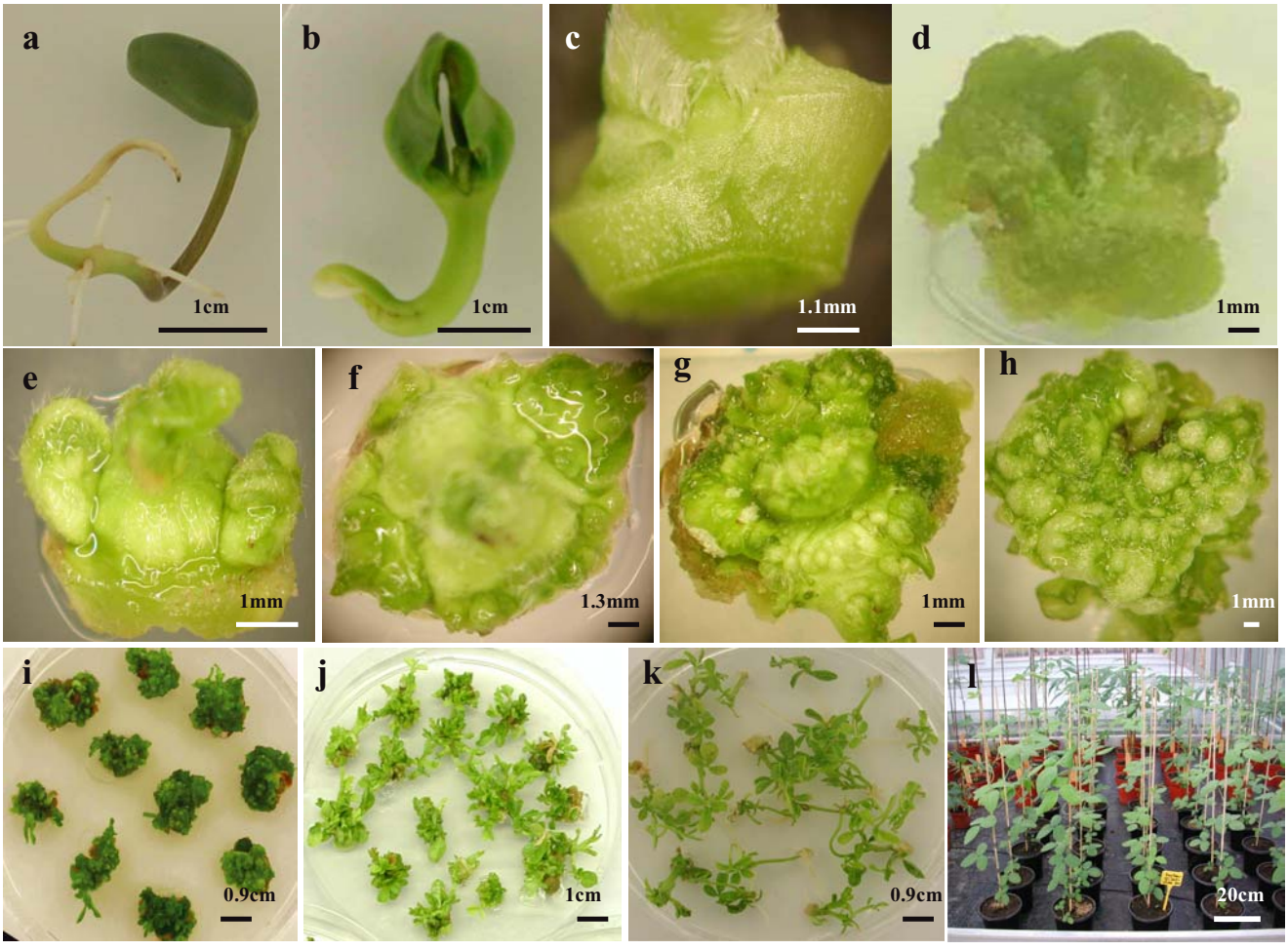

Fig. 1 Formation of multiple bud tissue (MBT), maintenance of MBT and plant regeneration in soybean: **(a)** seedling pretreated for 7 days on Murashige and Skoog (1962) salts and vitamins, 3% sucrose, 0.8% micro agar (MS), **(b)** seedling pretreated for 7 days on MS+0.1 mg/l TDZ, **(c)** cotyledonary node 7 days after germination (MS+0.1 mg/l TDZ), **(d)** compact callus formation on cotyledonary node without axillary buds, **(e)** enlargement of axillary bud (15 days after start of seed pretreatment), **(f)** formation of MBT (22 days after start of seed pretreatment), **(g)**, **(h)** formation of MBT (35 days after start of seed pretreatment), **(i)** maintenance of MBT, **(j)** shoot development of MBT, **(k)** rooting of shoots derived from MBT, **(l)** plant derived from MBT grown in 10 l pots in the greenhouse

Multiple bud tissue formation

The cotyledonary nodes, isolated from seeds pretreated on MS0 remained unchanged in size and morphology the following 3 weeks of culture on MS. When they were transferred to MS 0.5 mg/l BA, the axillary buds expanded and developed into two to three shoots and occasionally two or three new buds initiated from the regions adjacent to the axillary buds. Cotyledonary nodes isolated from seeds pretreated on MS 0.1 mg/l TDZ and cultured on MS0 formed one to four shoots and no new buds were produced.

Cotyledonary nodes isolated from seeds pretreated on medium supplemented with TDZ or BA and cultured on MS 0.5 mg/l BA or MS 0.1 mg/l TDZ expanded in size significantly and many new buds appeared from the regions adjacent to the axillary buds (Fig. 1e, f). The new buds were only formed if the cotyledonary nodes contained axillary buds. Removal of the axillary buds inhibited the formation of new buds and resulted in the production of excess callus (Fig. 1d) at both ends of the explants. This callus did not regenerate shoots. During culture continuously new buds were produced and they were arranged in discrete clusters (Fig. 1g, h) along the cotyledonary nodes. These tissues were defined as MBTs). The size of MBT varied with the seed pre-treatments. The cotyledonary nodes from 0.1 mg/l TDZ pre-treatment formed larger sections of MBT with higher numbers of buds than those from the other pretreatment media (Table 1).

The cotyledonary nodes isolated from seeds pretreated on MS 0.2 mg/l TDZ or MS 0.5 mg/l TDZ and cultured on MS 0.5 mg/l BA produced small sections of MBT, accompanied with non-regenerable callus. The MBT became vitrified during further subculture and only a few plants could be regenerated. Similar phenomenon was observed when cotyledonary nodes from MS 0.1 mg/l TDZ pretreatment were transferred to MS 0.1 mg/l TDZ. It was concluded that culture of cotyledonary nodes from MS 0.1 mg/l TDZ to MS 0.5 mg/l BA was the optimal way for MBT formation. The MBT from this treatment were used in the following experiments.

Table 2 The effect of different media on the maintenance of multiple bud tissue (MBT)

Medium used for MBT maintenance	Original weight (mg)	Weight after 2 weeks	Weight increase (%)	Composition of tissue after 2 weeks		
				Elongated shoots (%)	MBT[1] (%)	Compact callus (%)
MS 0.5 mg/l BA	63	430[b]	587	95	5[a]	0
MS 0.1 mg/l TDZ	63	463[b]	631	30	70[b]	0
MS 0.3 mg/l TDZ	62	377[a]	503	20	60[b]	20
MS 0.5 mg/l TDZ	59	456[b]	688	0	10[a]	90

Note. The data average of three replications (per replications 10 pieces of MBT of ±60 mg). MBT was produced by germinating seeds on MS 0.1 mg/l TDZ and transfer of the cotyledonary nodes to MS 0.5 mg/l BA. Values with different letters are statistically different ($p<0.05$) with the Fisher's protected LSD test

Multiplication of MBT

Multiple bud tissues were subcultured on MS 0.5 mg/l BA, MS 0.1 mg/l TDZ, MS 0.3 mg/l TDZ and MS 0.5 mg/l TDZ for multiplication. On all tested media the tissue increased about 6 times in fresh weight in 2 weeks. Depending on the medium, MBT either formed shoots or produced new MBT or formed compact calli (Table 2). On MS 0.5 mg/l BA, buds developed into shoots within 2–4 weeks and less new buds and shoots were formed with time. On MS 0.1 mg/l TDZ, a few buds developed into shoots, most MBT was multiplied without forming excess callus (Fig. 1i). On MS 0.3 mg/l TDZ, shoots and buds developed slowly, few buds developed into shoots, MBT became vitrified and compact callus was produced from the base of the tissue. This compact callus did not show any regeneration potential. On MS 0.5 mg/l TDZ, only compact callus was produced; MBT multiplication and shoot elongation were inhibited.

Based on the above described results MS 0.1 mg/l TDZ was chosen as medium for multiplication of MBT. Multiple bud tissues has been maintained on this medium for almost 1 year. When *in vitro* shoot nodes and shoot tips derived from MBT were cultured on MS 0.1 mg/l TDZ, all of them produced new MBT. Large-sized MBT was obtained after two or three cycles of culture. Shoot tips initiated much larger pieces of MBT than shoot nodes.

Plant regeneration

Multiple bud tissues maintained on MS 0.1 mg/l TDZ were transferred to media for shoot development. Buds developed into shoots within 6 weeks on MS 0.5 mg/l BA. Some of the explants produced shoots for a period of 3 months. The shoots were healthy and thick. One gram of MBT produced 336 shoots within 6 weeks (Fig. 1j). Less shoots developed from MBT cultured on MS 0.5 mg/l GA_3 (58 shoots/g). These shoots were thin and long and about 50% of the shoots became vitrified. Also the MBT of which the shoots were isolated became vitrified. After 2–4 more weeks of culture no new shoots were produced. Multiple bud tissues cultured on MS 0.2 mg/l BA produced 81 shoots/g and the shoots developed similarly as on GA_3 medium. Isolated single shoots were cultured for rooting on medium without growth regulators. More than 95% of the shoots produced roots within 4 weeks (Fig. 1k). Twenty randomly chosen plantlets were transferred to the greenhouse. All survived and grew normally (Fig. 1l). Because of the long-day conditions none of them set seeds in the greenhouse.

Discussion

The positive influence of pretreatment of seeds with TDZ or BA on regeneration of shoots has been reported before in soybean (Wright et al. 1986; Yoshida 2002) as well as in many other legumes such as pea, common bean, chickpea and lentil (Malik and Saxena 1992a, 1992b, 1992c, 1992d). In general pretreatment of seeds saves time and results in more shoots compared to the initiation from isolated organs such as hypocotyls or cotyledonary nodes (Malik and Saxena 1992a, 1992b, 1992c, 1992d).

In the system described here, the axillary buds are essential for MBT formation in soybean. Only the cotyledonary nodes with axillary buds gave 100% regeneration and produced MBT whereas those without axillary buds produced excess callus and no MBT. Wright et al. (1986) and Carmen et al. (2001) showed histologically that exogenously applied cytokinins altered the development of axillary meristems, promoted proliferation of the meristematic cells in the axillary buds and increased the number of bud primordia which originated from the existing axillary meristems. Malik and Saxena (1992c, 1992d) hypothesed that the high level of regeneration through TDZ or BA pretreatment of pea and common bean seeds was due to the morphological integrity of the seedlings. Our results suggested that the structural integrity of axillary meristem seemed to have contributed to the high efficiency of regeneration, since the shoots were regenerated from MBT, whose multiplication can be viewed as the proliferation of meristematic cells in the existing MBT. In many of the organogenesis systems described for soybean, the explants were taken from 2–14-days-old seedlings without cytokinin pretreatment. The regeneration frequency varied greatly with the explants used and between experiments. That might be due to the fact that dormant axillary buds are small and that the preparation of explants results in wounding of the axillary buds. Because dormant axillary buds start development after being transferred to a cytokinin supplemented medium, it takes more time to form shoots compared to axillary buds from cytokinin-pretreated seeds.

In the organogenesis system described here, the existing axillary buds are essential for MBT initiation. This is in contrast with others who have shown that shoots are regenerated from explants after removal of the axillary buds (Sairam et al. 2003). Maybe this can be explained by the different genotypes used.

For soybean organogenesis the explants are cultured first for 2–4 weeks on cytokinin supplemented medium for the initiation of shoots and then on elongation medium for growth of shoots. In all these systems meristems are initiated and then develop more or less linearly into shoots. This is different from the MBT system in which the development of meristems into shoots is slowed down at a certain moment and instead new meristematic cells are initiated in a repetitive way (repetitive organogenesis). As a result, very high multiplication rates are obtained. The fresh weight of the MBT increases 6 times in 2 weeks and and more than 300 shoots were produced from 1 g of MBT within a month. The shoot production in this research is much higher than that has been reported before for soybean. For example, Kaneda (1998) obtained 78 shoot/seed were obtained.

Soybean repetitive organogenesis has been reported before either by continuous culture on BA supplemented medium (Wright et al. 1987a, 1987b; Shetty et al. 1992) or by using alternate cycles of culture on TDZ and BA supplemented media (Barwale and Widholm 1990). However, in these repetitive organogenic systems the tissue was maintained in the multiple shoot stage. Seven shoots/explant were obtained when continuously cultured on BA supplemented medium every month and only 2% of the shoots developed into normal plants (Wright et al. 1987a, 1987b). No exact details were given when shoots were cultured on alternate cycles of BA and TDZ (Barwale and Widholm 1990). The MBT described here is maintained as a mixture of buds and shoots for a long period of time and more than 95% shoots develop into normal rooted plants. The MBT system has been tested in three other soybean genotypes (GL2051, GL270, and GL2705, kindly provided by the Germany Genebank). All formed MBT that was maintained by repeated subculture and produced large amounts of shoots, suggesting that the MBT system is genotype independent. However, the optimum TDZ concentration for MBT multiplication varies between genotypes (0.1–0.5 mg/l) and can be determined experimentally. When the TDZ concentration is too low, the shoots develop fast and there is no initiation of MBT. When the TDZ concentration is too high, shoots develop very slowly and there is abundant formation of compact callus.

Mature or immature seeds have been used as starting material in organogenesis and the meristems of the cotyledonary nodes as target tissue for soybean transformation (Christou et al. 1989, Finer and McMullen 1991). As meristematic cells represent only a small portion of the explants, the chance of transforming such cells is low. Moreover, the multicellular structure of meristems could cause formation of chimeric transgenic plants. The use of MBT could increase the efficiency of transformation since they contain much more meristematic cells than a single cotyledonary node. The repetitive subculture of MBT allows purification of completely genetically transformed tissue and subsequently plants from partially transformed meristems (Mathews et al. 1998). This approach has been successfully applied in pea where MBT infected with *Agrobacterium* and subcultured on TDZ supplemented medium resulted in transgenic plants (Tzitzikas et al. 2004) capable of transmitting the transgenes through seeds.

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