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ASSESSMENT OF THE INHIBITION ABILITY OF MANNOSE ON SOYBEAN SEED GERMINATION, SHOOT ELONGATION AND ROOTING FOR THE ESTABLISHMENT OF A MANNOSE SELECTION SYSTEM IN SOYBEAN TRANSFORMATION

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ABSTRACT

The inhibition ability of mannose on seed germination, shoot elongation and rooting of four soybean cultivars, MTD176, HL202, Williams 82, Bert was assessed by culture of seeds on the medium containing different concentrations of mannose: 0 (control), 25, 30, 35, 40, 45, 50, 55 g/l mannose. The results showed that germination rate started decreasing at 35 g /l mannose, while shoot elongation and rooting started to stop growth at 25 g/l mannose. At 55 g/l mannose, seed germination was entirely inhibited. Among the four test varieties, Bert was most sensitive to mannose. Based on these results, a selection system using mannose as the selective agent was tested for soybean transformation using Agrobacterium-mediated method. The cotyledons obtained from co-inoculation of cotyledonary nodes were subjected in three cycles with increasing mannose stepwise from 30, 35 and 40 g/l. The results showed that after 3rd selection, the non-transformed shoots stunted and died, while the transformed shoots could grow and elongate. GUS assay confirmed the expression of the gene transformed. The establishment of mannose selection system based on the use of the selectable marker gene, phosphomannose isomerase (pmi), would offer a novel approach in developing transgenic soybean cultivars, which are more environmental friendly, overcoming the constraints caused by using selectable marker gene encoding antibiotic or herbicide resistance.

Keywords:*Agrobacterium*-mediated transformation, mannose selection, phosphomannose isomerase (*pmi*), soybean

INTRODUCTION

The production of transgenic plant involves the use of a selectable marker gene to favor the regeneration of transformed shoots. In the traditional selection systems, these genes are either responsible for herbicide resistance or, most often, for antibiotic resistance (Olhoft *et al.* 2003). However, the presence of such genes may be undesirable in the final product.

A recent development has been based on the use of selectable marker genes, which give the transformed cells a metabolic advantage compared to the untransformed cells, which are starved with concomitant slow reduction in viability. This selection strategy is in contrast to the traditional selection where the non-transgenic cells are actively killed by the selective agent. Following this strategy, a selection system based on the selectable marker gene, phosphomannose isomerase (*pmi*) derived from *Escherichia coli* was developed. This selection system was proven to be successfully in producing transgenic plants from sugar beet (Joersbo *et al.* 1998), maize and wheat (Wright *et al.* 2001), japonica rice *cv.* Taipei 309 (Lucca *et al.* 2001), indica rice (Hoa and Bong 2003) and cotton (Hoa and Dung 2006).

In an effort to establish a selection system for soybean transformation using the selectable marker gene, *pmi* facilitating the use of mannose as the selective agent, the effect of mannose at different concentrations on the germination rate, shoot elongation and rooting of four soybean cultivars was assessed in this study. Based on this assessment, mannose concentrations effective in inhibiting soybean

shoot elongation and rooting were tested in selecting transgenic soybean shoots transformed by *Agrobacterium*-mediated method.

MATERIALS AND METHODS

Plant materials

Four soybean [*Glycine max* (L.) Merrill] cultivars, namely Bert, Williams 82, MTD176 and HL202 were used in this study. Bert, Williams 82 are introduced cultivars, while MTD176 and HL202 are the Vietnamese cultivars.

Medium culture and mannose treatment

The composition of germination medium (GM) is given in Table 1. Different concentrations of mannose as 25, 30, 35, 40, 45, 50 and 55 g/l were supplemented to the medium. The control contained 0 g/l mannose and 20 g/l sucrose.

Seed germination

Seeds from four test cultivars were surface sterilized by placing seeds into a tightly sealed chamber containing chlorine gas made by mixing 5 ml of 37% HCl (12N HCl) and 100 ml commercial bleach (5.25 % sodium hypochlorite) for 16 h. Sterilized seeds were germinated in 100 x 20 mm petri dishes, on the germination medium (GM, Table 1.) Fourteen seeds were served as one replication with four replications for each treatment. The plates were stacked 5 high and placed in plastic bags in which 4, approximately 3 inch slits were made with scissors. Seeds were germinated and grown in a growth chamber for 15 days at 25^0C under fluorescent lighting (90-150 mmol photons m^{-2} s^{-1}) in a 18/6 h (light/dark) photoperiod.

Assessment of germination rate, shoot and rooting

Germination rate was observed every day and recorded at day 8 and day 15 after placing seeds on GM medium. In this study, we define germination as the emergence of 2 mm or more of the radicle from the seed coat. The measurements of shoot elongation were recorded at day 15 for all the shoots emerging from each seed in the plates. Root elongation was recorded at day 15 for each shoot.

Transformation of soybean using mannose selection

The vector pManCa (Hoa and Bong 2003) containing the *pmi* gene and the *gus*A gene was used for transformation experiments. This binary vector was transformed into the competent cells of *A. tumefaciens* strain LBA 4404 (Hoekema *et al.* 1984). Cotyledonary nodes isolated from seeds after 5-7 days germination were used as explants. Transformation procedures using *Agrobacterium* methods were done basically as described by Olholf *et al.* (2003). After co-cultivation 5 days, the explants were maintained on the medium without mannose for 14 days; the cotyledons were excised from the callus/shoot pads and cultured on the medium containing 30 g/l mannose for first cycle selection. Second and third selections were done with 35 g/l mannose and 40 g/l mannose at 14-day intervals.

Elongated shoots after third cycle of selection were assessed for GUS activity following the procedures described by Jefferson *et al.* (1987).

Table 1. Components of germination medium (GM) and mannose treatments used to assess the inhibition of mannose on soybean seed germination, shoot elongation and rooting.

GM medium	Treatment							
	Control	T1	T2	T3	T4	T5	T6	T7
B5 salts[a]	1X	1X	1X	1X	1X	1X	1X	1X
MS iron stock	1X	1X	1X	1X	1X	1X	1X	1X
B5 vitamins	1X	1X	1X	1X	1X	1X	1X	1X
Sucrose (g/l)	20	-	-	-	-	-	-	-
Mannose[b], (g/l)	-	25	30	35	40	45	50	55
Phytagel[c] (g/l)	3	3	3	3	3	3	3	3
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8

[a]Duchefa, Netherlands; [b]Acros, USA; [c]Sigma, USA.

RESULTS AND DISCUSSION

Inhibition of mannose on soybean seed germination, shoot elongation and rooting

Free mannose is not found in green plants except in trace amounts in some species (Herold and Lewis 1997). In the culture medium supplemented with mannose, mannose absorbed in the cells is converted to mannose-6-phosphate by endogenous hexokinase, which accumulates resulting in severe growth inhibition since the plants cannot metabolize mannose-6-phosphate. Hexokinase is likely to be the first component of the pathway. This was reported by Graham *et al.* (1994) in cucumber and by Jang and Sheen (1997) and Jang *et al.* (1997) in maize and *Arabidopsis*.

In this study, the inhibition ability of mannose on soybean seed germination, shoot elongation and rooting were assessed.

Seeds from the four cultivars *viz* Bert, Williams 82, MTD176 and HL202 were cultured onto the medium containing different concentrations of mannose as 25, 30, 35, 40, 45, 50 and 55 g/l. The results (Figure 1) showed that at 25, 30 and 35 g/l, germination rate of the cultivars MTD176, HL202 and Bert was 100% as the control, which did not contain mannose. At 40 mg/l mannose, germination rate of these cultivars started decreasing. Seed germination of the cultivar Williams 82 started to decrease at a lower concentration, i.e. 35 g/l. The decrease in germination rate of Bert and HL202 was significantly affected at 45 and 50 g/l mannose, respectively. It was recorded that no seeds of Bert germinated at 50 g/l, while the three other cultivars failed germination at 55 g/l indicating that Bert was very sensitive to mannose. MTD176 and Williams 82 were less sensitive than Bert and HL202. The increase of mannose seemed to delay the start of seed germination; however, if seeds did not germinate after 7 days on GM medium, they would not germinate afterward.

In contrast to germination rate, shoot elongation started a sharp decrease at 25 g/l mannose with a length of around 3 cm as compared to 15-17 cm in the control (Figure 2 and 3). The length of shoots of all cultivars decreased further as mannose concentration increased. At 50 g/l three cultivars, MTD176, HL202 and Williams 82 had very stunted shoots being below 2 cm, while the cultivar Bert failed to develop shoots.

It was observed that mannose suppressed rooting of shoots (Figure 3A, 3D). As compared to the control, rooting suppression was easily seen at 30 g/l mannose and above with only few shoots to have roots. However, the roots of these seedlings started browning and died after three weeks. At 50 g/l mannose, lateral roots of all the cultivars could not be formed.

The effect of mannose on seed germination rate and shoot elongation of cotton cultivars was investigated by Hoa and Dung (2006) showing that at 15 g/l mannose, seed germination rate started to decrease and

after 2 weeks, shoots were dead. At 35 g/l mannose, up to 98% of shoots were dead. It seemed that cotton is more sensitive to mannose than soybean.

Transformation of soybean using mannose selection

To produce transgenic soybeans, selection systems that produced transformed plants have employed the selectable marker genes, neomycin O-phosphotransferase (*npt*II), phosphinothricin acetyltransferase (*bar*), or 5-enolpyruvylshikimate-3- phosphate synthase (*epsps*), in combinations with the selective agents kanamycin (Hinchee *et al.* 1988; Di al. 1996), glufosinate [phosphinothricin (PPT); Zhang *et al.* 1999], and glyphosate [N-(phosphonomethyl) glycine; Clemente *et al.* 2000], respectively. The development of transgenic plants containing selectable marker genes for antibiotic or herbicide resistance has caused widespread public concern on the biosafety of genetically modified organisms. To overcome this constraint, alternatively, a novel selective marker gene, phosphomannose isomerase (*pmi*) derived from *Escherichia coli* was recently used in plant transformation. The *pmi* gene encodes for phosphomannose isomerase, which is able to convert mannose-6-phosphate, an unusable carbon source for most plant cells, into fructose-6-phosphate- a carbohydrate source that can be utilized by the plant tissue. Therefore, the transformed plants containing the *pmi* gene either can grow normally on the medium supplemented with mannose, while the non-transformed plants stops growing or dies due to starvation (Hansen and Wright 1999).

Based on the results on assessing the effect of mannose on soybean seed germination, shoot elongation and rooting, the selection by mannose was established and tested for soybean transformation, using *Agrobacterium* method with the vector pManCa carrying *pmi* and *gus*A genes.

The cotyledons obtained from co-cultivation of cotyledonary nodes were subjected in three cycles with increasing mannose stepwise from 30, 35 and 40 g/l. The results showed that after third selection, the non-transformed shoots stunted and died, while the transformed shoots could grow and elongate (Figure 4). GUS assay confirmed the expression of the gene transformed (Figure 5). The establishment of mannose selection system based on the use of the selectable marker gene, *pmi* would be of significance in developing transgenic soybean cultivars, which are more environmental friendly.

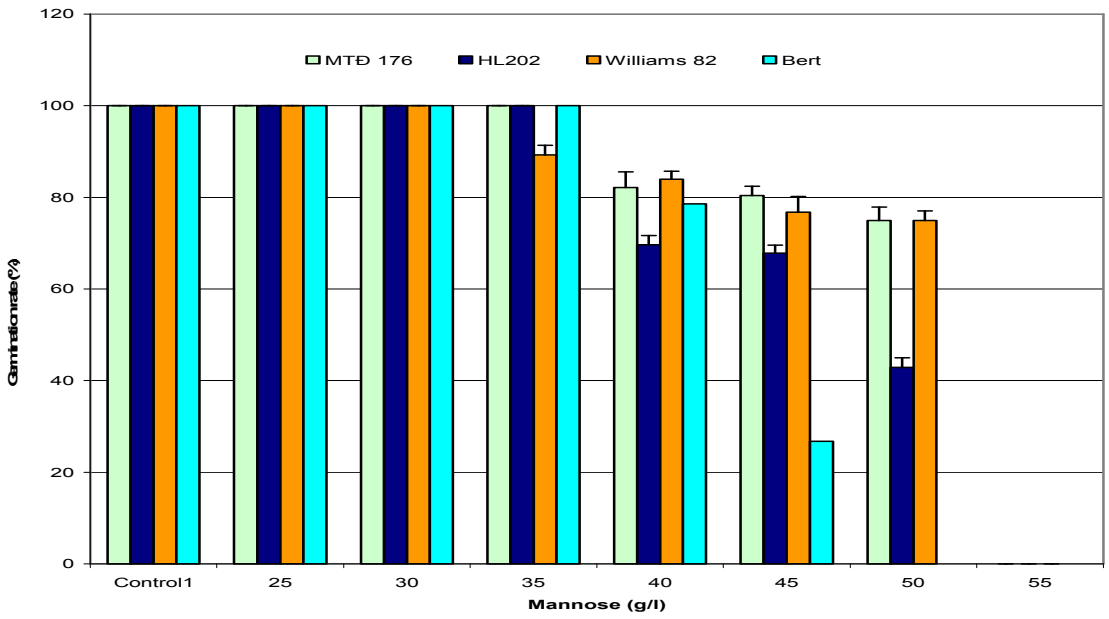

Figure 1. Effect of mannose on germination rate of 4 soybean cultivars (MTD176, HL202, Williams 82, Bert), mannose concentrations: 0 (control), 25, 30, 35, 40, 45, 50, 55 g/l.

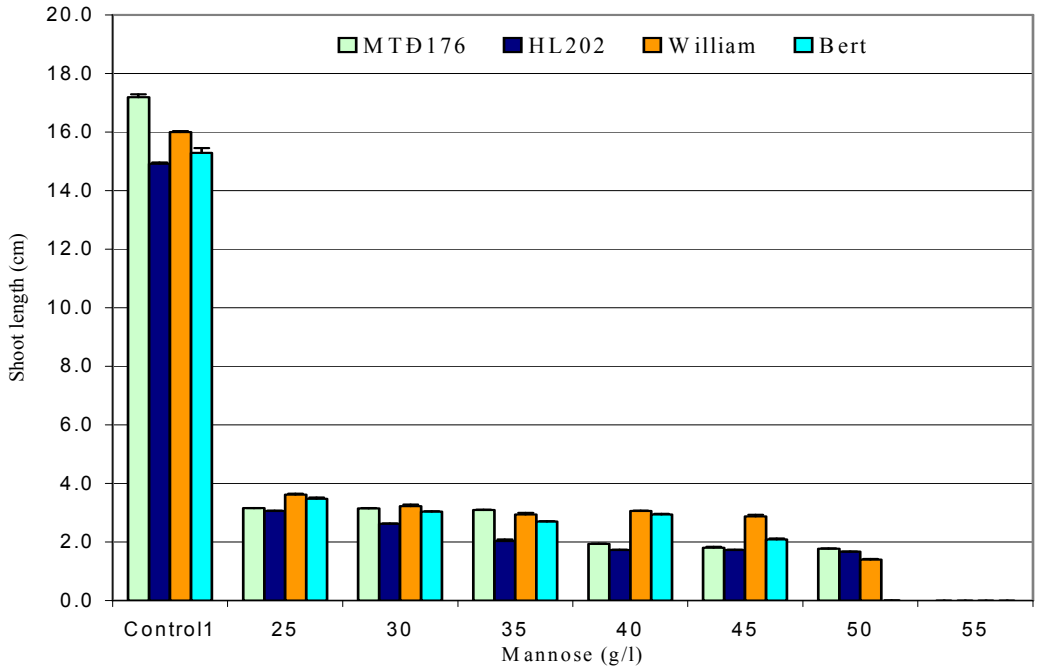

Figure 2. Effect of mannose on shoot length of 4 soybean cultivars (MTD176, HL202, Williams 82, Bert), mannose concentrations: 0 (control), 25, 30, 35, 40, 45, 50 and 55 g/l.

Figure 3A. Effect of mannose on shoot elongation and rooting of the soybean cultivar Bert, mannose concentrations: 0 (control), 25, 30, 35, 40, 45, 50 g/l.

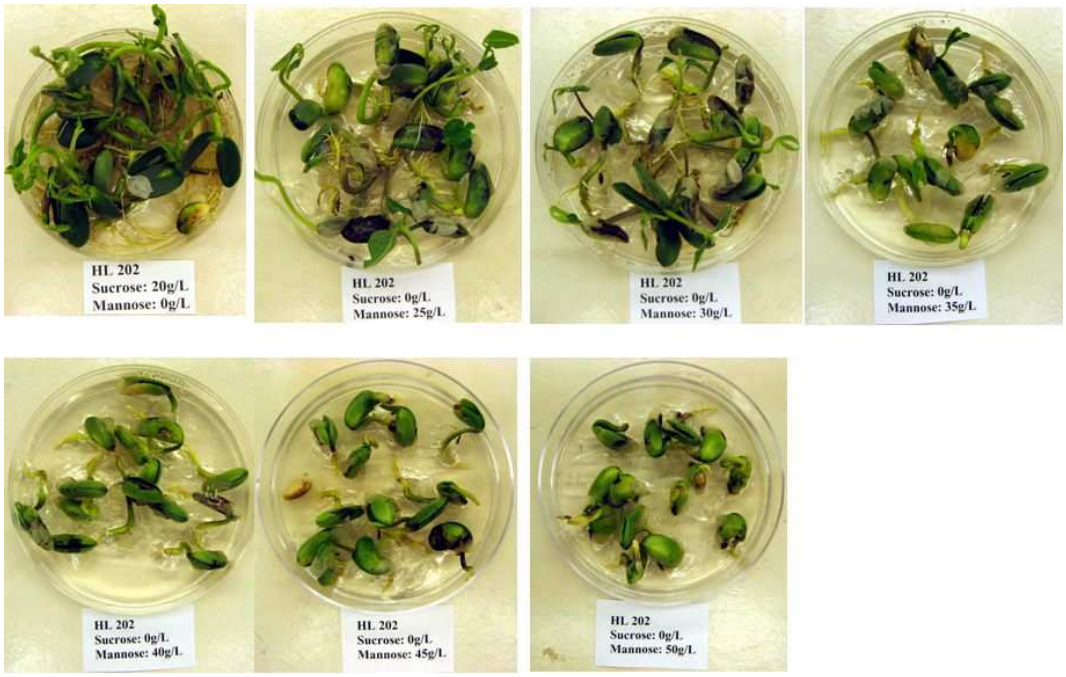

Figure 3B. Effect of mannose on shoot elongation and rooting of the Vietnamese soybean cultivar HL202, mannose concentrations: 0 (control), 25, 30, 35, 40, 45, 50 g/l.

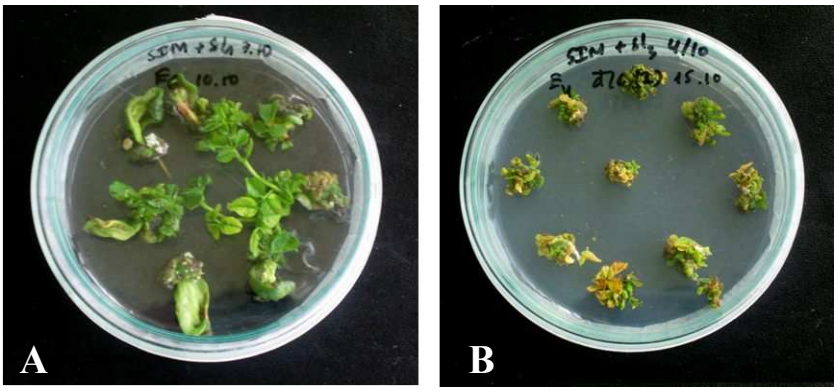

Figure 4. Mannose selection (3rd cycle) A: transformed shoots survived and elongated B: non-transformed shoots stunted and died

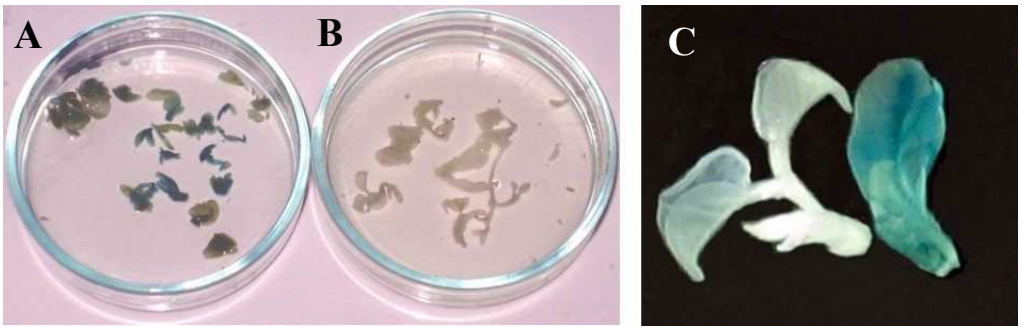

Figure 5. GUS assay of soybean shoots transformed with pManCa containing *pmi* and *gus*A gene, and selection by mannose (A: young shoots showing GUS^+, B: non-transformed shoot, C: left: non-transformed shoot, right: transformed GUS^+ shoot

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Đánh giá khả năng ức chế của mannose đối với tỷ lệ nẩy mầm, phát triển chồi và rễ của đậu nành để xây dựng hệ thống chọn lọc bằng mannose cho chuyển nạp gen ở đậu nành

Khả năng ức chế của mannose đối với tỷ lệ nẩy mầm, phát triển chồi và rễ của bốn giống đậu nành: MTĐ176, HL202, Williams 82, Bert được đánh giá bằng nuôi cấy hạt trên môi trường có chứa mannose với các mức nồng độ khác nhau: 0 (đối chứng) 0, 35, 40, 45, 50, 55 g/l mannose. Kết quả cho thấy, tỷ lệ nẩy mầm bắt đầu giảm ở nồng độ 35 g/l mannose, trong khi phát triển chồi và rễ ngừng ở 25 g/l mannose. Sự nẩy mầm hoàn toàn bị ức chế ở nồng độ 55 g/l mannose. Trong bốn giống thử nghiệm, Bert là giống mẫn cảm với mannose nhất. Căn cứ các kết quả này, một hệ thống chọn lọc dùng mannose là tác nhân chọn lọc được thử nghiệm trong chuyển nạp gen ở đậu nành bằng phương pháp *Agrobacterium.* Cây mầm phát triển từ các nốt lá mầm sau khi chủng, được tiến hành qua ba vòng chọn lọc với nồng độ mannose tăng dần qua mỗi vòng từ 30, 35 đến 40 g/l. Kết quả cho thấy: sau vòng chọn lọc thứ 3, các chồi đối chứng không chuyển gen không phát triển được và chết, trong khi các chồi được chuyển gen phát triển, vươn lóng bình thường. Thử nghiệm GUS cho thấy sự biểu hiện của gen chuyển nạp. Việc xây dựng hệ thống chọn lọc mannose nhờ vào việc sử dụng gen đánh dấu chọn lọc phosphomannose isomerase (*pmi*) mở ra hướng mới trong tạo giống đậu nành biến đổi gen thân thiện hơn với môi trường, khắc phục hạn chế do việc sử dụng các gen đánh dấu chọn lọc tạo tính kháng chất kháng sinh hoặc kháng chất trừ cỏ.