PROPAGATION OF *Plantago major* L. BY PLANT TISSUE CULTURE TECHNIQUE

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ABSTRACT

Plantago major L., a species that originated in the Far East, has been used for medicinal aspects since ancient times. The technique for callus induction, growth, and plant regeneration from cultured young leaves and petiole explants of *Plantago major* was carried out. The best initiation and growth of calli was achieved on MS supplemented with 1 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BA. Shoot regeneration from the calli was obtained on MS medium containing 1 mgL^{-1} TDZ alone or combined with 0.5 mgL^{-1} α-NAA (100 and 96%, respectively). Moreover, the optimum medium for shoot multiplication was MS medium comprising 5 mgL^{-1} BA, and root formation from the shoots was half-strength MS medium supplemented with 2 mgL^{-1} NAA. The regenerated plants were successfully transferred into pots containing a mixture of decayed straw: rice husk ashes, (1:1, v/v), sand: soil (1:1, v/v), soil, or sand and grown in nethouse. The phenotypes of regenerated plants were normal as a mother plant.

Keywords: 2,4-D: 2,4-Dichlorophenoxyacetic acid, ABBREVIATIONS, Acclimatization, BA: Benzyl adenine, NAA: α-Naphthaleneacetic acid, MS: Murashige and Skoog, *Plantago major* L., plant growth hormone, TDZ: Thidiazuron, tissue culture.

Nhân giống cây mã đề (*Plantago major* L.) bằng kỹ thuật nuôi cấy mô

TÓM TẮT

Cây mã đề (*Plantago major* L.) có nguồn gốc từ miền Viễn Đông, là loại dược liệu được sử dụng từ xa xưa. Kỹ thuật tạo và nuôi cấy mô sẹo cũng như tái sinh chồi từ cuống lá và lá non cây mã đề *P. major* L. đã được thực hiện. Kết quả nghiên cứu cho thấy: (i) môi trường thích hợp cho sự tạo mô sẹo là môi trường MS bổ sung 2,4-D 1 mg/L kết hợp với BA 0,5 mg/L; (ii) Từ các mô sẹo hình thành, có thể tái sinh chồi cây mã đề trên môi trường MS bổ sung TDZ 1 mg/L hoặc TDZ 1 mg/L kết hợp với NAA 0,5 mg/L sẽ cho hiệu quả cao (tương ứng 100 và 96%); (iii) Từ các chồi tái sinh có thể cấy truyền trên môi trường MS bổ sung BA nồng độ từ 5 mg/L nhằm gia tăng số chồi; (iv) Trong giai đoạn tạo rễ *in vitro*, có thể sử dụng môi trường MS bổ sung NAA nồng độ 2 mg/L. Cây con sau khi tạo rễ đều sinh trưởng, phát triển tốt và có hình thái như cây mẹ trên các loại giá thể: phân rơm mục + tro trấu (tỷ lệ 1:1), cát + đất (1:1); đất hoặc cát khi thuần dưỡng trong nhà lưới.

Từ khóa: Cây mã đề, chất điều hòa sinh trưởng, nuôi cấy mô, *Plantago major* L., thuần dưỡng.

1. INTRODUCTION

The investigation of medicines of plant origin is prospective of the current trends in medical research because of their affordability and accessibility with minimal side effects. Among the medicinal plants, plantain (*Plantago major* L.), belonging to the Plantaginaceae family, is considered to be a potential herb because it contains high levels of biosubstances such as iridoid glycosides, polysaccharides, flavonoids, and some organic acids that are involved in wound healing activity, and have anti-inflammatory, analgesic, antioxidant, weak

antibiotic, immune modulating, antiulcerogenic, antileukemic, and antihypertensive activity effects (Ho, 2000; Kolak *et al.*, 2011). This species has attracted much attention and has become economically important (Basma *et al.*, 2012).

Conventional propagation methods, such as seeds, grafting, cuttings, and suckers, are in place but are cumbersome and season dependent. Therefore, the application of modern tools of biotechnology needs to be standardized for harnessing the maximum benefits from this medicinal plant (Kalia *et al.*, 2011). The plant tissue culture technique is one of many valuable tools in plant breeding programs. Compared to other vegetative methods, it is a convenient technique for producing clonally uniform and genetically identical, axenic plants of many desirable plant species. This technique is convenient and facilitates year-round production capability and requires less space (IAEA, 2004). It has immense potential in mass propagation in a genetic improvement program of *Plantago major*. Indeed, plantain propagation through conventional and tissue culture methods were successfully achieved for several species: *P. lanceolata* L., *P. major*, *P. maritima* L., *P. media* L. and *P. ovata* Forssk (Tu, 1996; Makowczynska and Golec, 2000; Makowczynska and Golec, 2003; Fons *et al.*, 2008). However, callus induction or direct or indirect shoot regeneration is still limited (Fons *et al.*, 2008). Positive results regarding Mongolian and Vietnamese broadleaf plantain propagation has not yet been studied. This study was conducted with the aims of determining the optimal media for regeneration of plantain plants by the plant tissue culture technique in order to apply for and improve plantain production and application, as well as contributing to the development of the broadleaf plantain processing industry that supplies products for both domestic and overseas consumptions.

2. MATERIALS AND METHODS

2.1. Plant materials

The matured seeds of *P. major* were surface-sterilized by rinsing with 70% ethanol for 2 min and rinsed 3 times in sterile distilled water. Then the seeds were sterilized in 2% (v/v) NaOCl and 0.03% (v/v) Tween 20 for 15 minutes, and rinsed 3 times in sterile distilled water. Then the seeds were rinsed by 0.1% (w/v) $HgCl_2$ solution for 2 minutes and finally rinsed 6 times in sterile distilled water.

2.2. Media and culture conditions

All samples were plated on a medium comprised of basal salts and vitamins of MS (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, and 0.2% (w/v) phytagel. The medium was adjusted to pH 5.8, and then autoclaved at 1.2 - 1.3 kg/cm^2 pressure and 121°C for 20 min. The cultures were maintained under *in vitro* culture conditions adjusted to 25 ± 1°C and 16h photoperiod of 45 µmol m^{-2}s^{-1} illumination provided by cool white fluorescent lamps. After 10 days of culture, explants (5 mm in diameter of young leaves, 5 mm in length of petioles) were aseptically excised from *P. major* seedlings and used for further experiments. All cultures were maintained under the same *in vitro* culture conditions as described above.

2.2. Methods

2.2.1. Effect of various hormone concentrations and combinations on callus induction

To determine the effect of growth regulators on callus induction from the young leaf and petiole explants, MS media containing various combinations of 2,4-D concentrations (0, 0.1, 0.5 and 1 mgL^{-1}) and BA concentrations (0, 0.5, 1 and 2 mgL^{-1}) were used. Each treatment was carried out in 5 replicates (10 explants in a Petri dish per replication) for each type of sample. After 4 weeks of culture, the percentage of callus induction was counted.

2.2.2. Shoot regeneration and multiple shoots

Callus clusters were selected from the best treatment of the callus induction experiment above to be used for further analyses. To determine the effect of growth regulators on shoot regeneration, MS media containing various combinations of NAA concentrations (0, 0.5 and 1 mgL^{-1}) and TDZ concentrations (0, 0.2, 0.5 and 1

mgL^{-1}) were used. Each treatment was carried out in 5 replicates, 10 explants in a Petri dish per replication. After 4 weeks of culture, the percentage of shoot regeneration was counted.

To evaluate the effect of BA concentration on shoot multiplication, shoot tips with 3-4 leaves were cultured. The MS media containing various BA concentrations (0, 1, 2, 3, 5 and 7 mgL^{-1}) were used.

Each treatment was carried out with 5 replicates, 3 explants in a jar per replication. After 2, 4, 6 and 8 weeks of culture, the number of new shoots was counted.

2.2.3. Root induction

Regenerated shoots, without roots, were transferred into MS medium (half and full strength) supplemented with activated charcoal 2 gL^{-1} and various concentrations of NAA (0, 0.5, 1, 2 and 4 mgL^{-1}) to test the effect of the hormone NAA on rooting. After 4 weeks of culture, the numbers of regenerated roots were counted.

2.2.4. Acclimatization

After root formation, the plants were transferred to 20 × 20 cm plastic pots with 4 substrates including sand, soil, sand: soil (1:1, v/v), and decayed straw: rice husk ashes (1:1, v/v). The experiment was carried out with 4 treatments, 5 replications each, and 15 plantlets per replication.

2.3. Statistical analysis

All the data were analyzed using the statistical package for social sciences (version 16.0 for Windows, SPSS Inc.) and Microsoft Excel software to perform ANOVA and DUNCAN tests ($P \leq 0.05$ or 0.01).

3. RESULTS AND DISCUSSION

3.1. Effect of various hormone concentrations on callus induction of plantain

Results from the callus induction experiment clearly indicated that the percent of calli formed was significantly different at the 0.01 level. The highest percentage of callus induction was on the MS medium containing 1 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BA (Table 1). However, there was not a significant difference on the type of samples or interaction between sample types and hormone combination on the callus induction. This result is analogous to research on *P. major* growth which studied MS media containing 0.5-1 mgL^{-1} 2,4-D combined 0.2 mgL^{-1} BAP (Tu, 1996; Makowczynska and Andrzejewska-Golec, 2000).

3.2. Effect of various hormone concentrations and combinations on shoot organogenesis

Shoot primordia were formed at the base of the callus explants within two weeks of culturing. These primordia differentiated into green shoots with 2-3 foliar appendages after a period of 4 weeks. The effect of various MS media on shoot induction from the callus clusters is presented in Figures 1, 2B & F. It was observed that the response to TDZ concentration at 1 mgL^{-1} or combination with 0.5 mgL^{-1} NAA was statistically different ($P < 0.01$) with the highest percentage of explants producing shoots, 100 and 96% shoot induction, respectively.

However, the shoot organogenesis was not formed on the other combinations of NAA and TDZ concentrations. In this study, TDZ was more effective at inducing shoot regeneration from calli of *P. major* than in combinations with NAA. TDZ is a substitute phenyl urea that shows cytokinin-like activity similar to that of the N_6 substituted adenine derivatives (Mok *et al.*, 1982). TDZ has been shown to induce the accumulation of both endogenous auxins and cytokinins in legumes and herbaceous species (Murthy *et al.*, 1995; Hutchinson and Saxena, 1996). Also, treatment of soybean calli with TDZ stimulates cytokinin accumulation (Thomas and Ketterman, 1986). This is probably because cytokinins are inactivated irreversibly by two mechanisms, one of which is the oxidative cleavage of the N_6 side chain of the cytokinin substrate by cytokinin oxidase (Kende and Zeevaart, 1997). TDZ is known to non-competitively inhibit cytokinin oxidase activity (Chatfield and Armstrong, 1986), thereby enhancing the availability of endogenous cytokinins. In this study also, TDZ displayed more effective shoot regeneration ability than other hormones, supporting these previous hypotheses.

Table 1. Effect of hormone combination and sample types on callus *Plantago major*, %

Hormone combination (mgL^{-1})	Type of samples (%, ± SD)		Callus induction, % (A)
	Young leaf	Petiole	
Control (hormone free MS)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0) e
0.5 BA	0.0 (± 0.0)	0.0 (± 0.00)	0.0 (± 0.0) e
1 BA	0.0 (± 0.00)	0.0 (± 0.0)	0.0 (± 0.0) e
2 BA	0.0 (± 0.00)	0.0 (± 0.0)	0.0 (± 0.0) e
0.1 2,4-D	6.7 (± 0.00018)	6.7 (± 0.00018)	6.7 (± 0.00017) e
0.1 2,4-D + 0.5 BA	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0) e
0.1 2,4-D + 1 BA	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0) e
0.1 2,4-D + 2 BA	6.7 (± 0.00018)	6.7 (± 0.00018)	6.7 (± 0.00018) e
0.5 2,4-D	46.7 (± 0.0107)	46.7 (± 0.0107)	46.7 (± 0.01) cd
0.5 2,4-D + 0.5 BA	53.3 (± 0.00107)	40.0 (± 0.00115)	46.7 (± 0.00107) cd
0.5 2,4-D + 1 BA	26.7 (± 0.00099)	33.3 (± 0.00093)	30.0 (± 0.0009) de
0.5 2,4-D + 2 BA	46.7 (± 0.0011)	46.7 (± 0.0119)	46.7 (± 0.0107) cd
1 2,4-D	73.3 (± 0.00242)	60.0 (± 0.00224)	66.7 (± 0.00227) b
1 2,4-D + 0.5 BA	86.7 (± 0.00193)	93.3 (± 0.00158)	90.0 (± 0.00170) a
1 2,4-D + 1 BA	66.7 (± 0.00198)	53.3 (± 0.00107)	60.0 (± 0.00160) bc
1 2,4-D + 2 BA	46.7 (± 0.00107)	46.7 (± 0.00106)	46.7 (± 0.00100) cd
Callus induction on sample types (B, %)	28.8 (± 0.00167)	27.0 (± 0.00157)	
P_A	**		
P_B	ns		
P_{A*B}	ns		

*Note: Means (± SD) of five replicates (Petri dishes) with 10 explants each. Means with same letters indicate no significant difference by Duncan's multiple-range test; ns: not significant; ** = significant at 0.01 level*

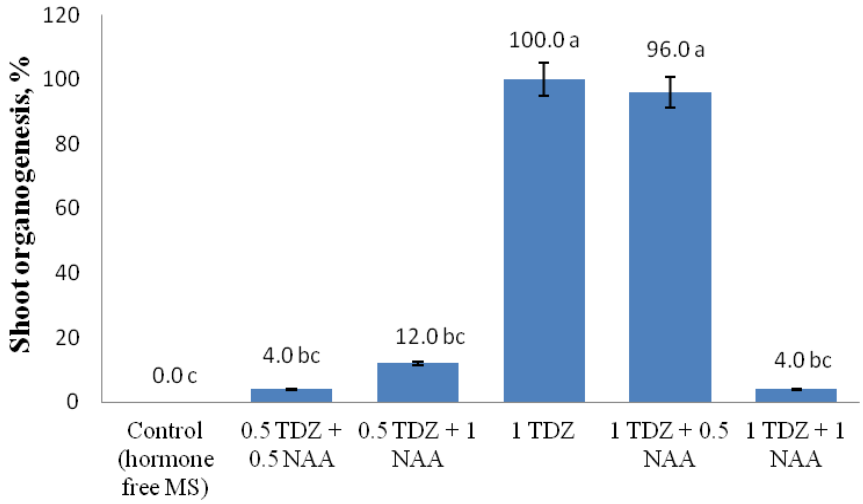

Figure 1. Effect of different hormone concentrations and combinations on the percentage of shoot organogenesis of *P. major* at four weeks after culture

*Note: Means (± SD) of 5 replicates with 10 explants per each Petri dish. Means with same letters indicate no significant difference by Duncan's multiple-range test; **: significant at 0.01 level*

Table 2. Effect of BA concentration on number of *in vitro* proliferated shoot of plantain

BA concentration (mgL^{-1})	Number of shoot (± SD, weeks after cultured)			
	2	4	6	8
Control (hormone free MS)	1.0 (± 0.0) c	1.2 (± 0.2) c	1.4 (± 0.1) c	1.5 (± 0.2) c
1	1.5 (± 0.3) bc	1.7 (± 0.3) c	2.2 (± 0.7) b	2.5 (± 0.6) b
3	1.7 (± 0.5) b	2.5 (± 0.7) b	2.7 (± 0.5) b	3.0 (± 0.6) b
5	2.1 (± 0.7) ab	3.2 (± 0.5) a	3.6 (± 0.6) a	4.7 (± 0.3) a
7	2.7 (± 0.6) a	3.6 (± 0.3) a	4.0 (± 0.2) a	4.7 (± 0.6) a
P	**	**	**	**

*Note: Means (± SD) of 5 replicates (jars) with 3 explants each. Means with same letters indicate no significant difference by Duncan's multiple-range test; **: significant at 0.01 level*

Table 3. Effect of NAA concentration on number of *in vitro* induced root of plantain

NAA concentration (mgL^{-1})	Number of root (root/plant, ± SD)		Number of root (± SD, A)
	Full strength MS medium	Half strength MS medium	
0	5.7 (± 1) d	8.46 (± 1.4) c	7.0 (± 1.8) d
0.5	10.0 (± 1.9) bc	12.4 (± 2.3) b	11.2 (± 2.4) c
1	8.9 (± 2.4) c	17.7 (± 1.2) a	13.3 (± 4.9) b
2	15.7 (± 1.2) a	15.7 (± 1) a	15.7 (± 1.1) a
4	8.4 (± 4) c	15.1 (± 1) a	11.7 (± 4) bc
Number of root on media types (B)	9.7 (± 4) b	13.8 (± 3.5) a	
P_A	**		
P_B	**		
P_{A*B}	**		

*Note: Means (± SD) of 5 replicates (jars) with 3 explants each. Means with same letters indicate no significant difference by Duncan's multiple-range test; **: significant at 0.01 level*

3.3. Effect of BA concentration on multiplication of plantain

The effect of various BA concentrations on shoot proliferation from the explants is presented in Table 2. It was observed that the response to a range of concentrations of BA was statistically different ($P < 0.01$) with respect to the number of shoots per explants after eight weeks cultured. New shoots formed on the MS medium with 5 or 7 mgL^{-1} BA. Those shoots were healthier (Fig. 2C & G) and could be readily rooted. They were multiplied on a similar medium for over six months and the shoots obtained in this way were noticed to be normal. This present result was equivalent to the others on *P. asiatica* L. (Tu, 1996), *P. major* (Li and Li, 2005).

3.4. Effect of NAA concentration on rooting induction of plantain

The combinations of NAA (0, 0.5, 1, 2 and 4 mgL^{-1}) with MS media (full and half strength) were tested to optimize the concentration of the growth regulator and minerals for rooting.

According to Makowczynska and Andrzejewska-Golec (2003), although *P. major* could be rooted on MS medium without plant growth regulators, in our results, we observed the highest root number was on half strength MS containing 1 mgL^{-1} NAA, and this combination was significant higher than the other treatments at the 0.01 level, except the half strength MS supplemented with 2 mgL^{-1} NAA and 4 mgL^{-1} NAA or full strength MS plus 2 mgL^{-1} NAA treatments (Table 3). No callusing or browning phenomenon of the samples was observed during the root formation stage. It has been reported that different cultivars do not respond in the same way during establishment, proliferation and rooting *in vitro* (Van Huylenbroeck and Debergh, 1996). As listed in Table 3, half strength MS medium was more effective than full strength MS for root induction, 13.8 roots and 9.7 roots, respectively. The statistical difference was at 0.01 level. The results showed that the chemical investment can be reduced significantly. Optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirements. Tissues from different parts of plants may also have different requirements for satisfactory growth (George, 1993). In the *in vitro* rooting stage, plantlet quality may be effectively improved by reducing the macro mineral requirements, especially lowering nitrogen concentration (Driver and Suttle, 1987).

3.5. Effect of different substrates of regenerated plants

The ultimate success of micropropagation on a commercial scale depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates (Chandra *et al.*, 2010). Bekman and Lukens (1997) mentioned that the supporting material has a crucial role and could influence the survival percentage, growth and development in the acclimatization stage. The results showed that the survival rate and new number of leaves and shoots were highest with all substrates, with no significant difference at P = 0.05 (Table 4). High percentages of plantlets were successfully transferred into potted soil and developed into normal plants in the greenhouse with 93.3-100% survival after four weeks. No phenotypic variability in morphology in the plants was observed (Fig. 2). *In vitro* culture of *P. major* can support plant material for phytochemical analysis. Thus, regenerated shoots of this plant may contain such crucial important biosubstances as iridoid and other glycosides (Samuelsen, 2000; Pourmorad *et al.*, 2006; Souri *et al.*, 2008).

Callus induction, shoot regeneration, and multiplication from young leaf (A, B, C) and petiole explants (E, F, G), respectively. D: Rooted shoot after 4 weeks of culture on MS medium supplemented with 1-4 mgL^{-1} NAA. H: Micropropagated *P. major* plant after 4 weeks of pot growth.

4. CONCL.USION

We established an efficient regeneration procedure for Plantago major and found that the petioles or young leaves of immature stems induced the highest ratio of calli induction and compact calli formation on MS medium supplemented with 1 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BA after 4 weeks of culture. From regenerated calli, somatic embryogenesis could be induced on MS medium supplemented with 1 mgL^{-1} TDZ or 1 mgL^{-1} TDZ and 0.5 mgL^{-1} NAA. The best medium for shoot proliferation was MS medium supplemented with BA 5 mgL^{-1}; while solid MS medium supplemented with 2 gL^{-1} activated charcoal and 2 mgL^{-1} NAA was the most effective for root induction of plantain. The regenerated plantlets were successfully transferred into pots containing a mixture of decayed straw: rice husk ashes, (1:1, v/v), sand: soil (1:1, v/v), soil, or sand. Plantlets showed a high survival rate and almost plantlets grew normally in the nethouse conditions.

Table 4. Effect of different substrates on the acclimatization of micropropagated *Plantago major* after 4 weeks of cclimatization

Substrates	Survival (%, ±SD)	No. of new leaves (±SD)
Sand	93.3 (± 25.2)	2.3 (± 0.9)
Soil	100 (± 0.0)	2.4 (± 0.7)
Sand: Soil (1:1, v/v)	100 (± 0.0)	2.57 (± 0.6)
Decayed straw: rice husk ashes (1:1, v/v)	100 (± 0.0)	2.6 (± 0.4)
P	ns	ns

Note: Means (± SD) of 5 replicates with 15 plantlets each; ns: not significant

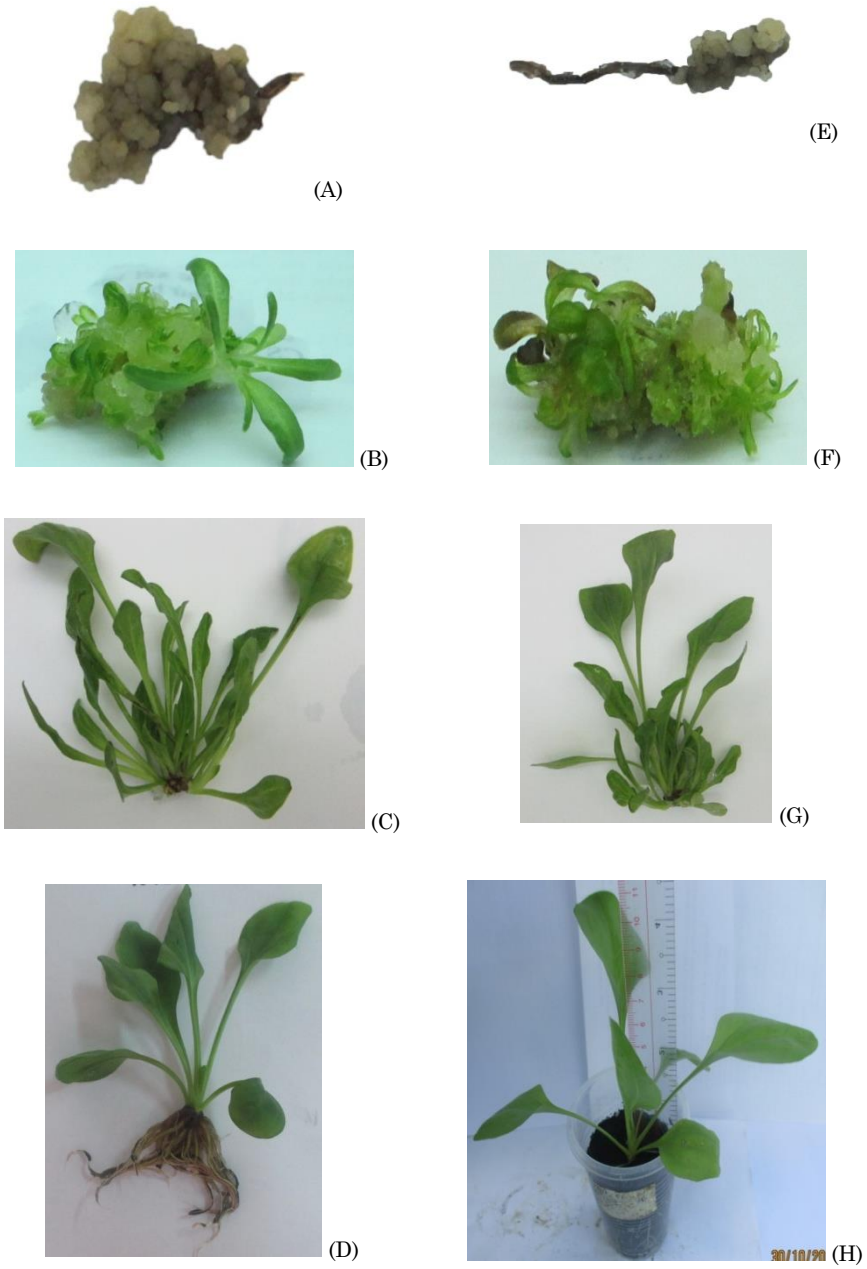

Figure 2. Regeneration of *Plantago major*

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