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Full Length Research Paper

Plant regeneration system from cotyledons-derived calluses cultures of *Stylosanthes guianensis* cv. 'Reyan 2'

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The objective of this study was to successfully establish plant regeneration system with cotyledons of *Stylosanthes guianensis* Sw. cv. 'Reyan 2' as explants. In this study, the following results were obtained; (1) the highest rates of callus induction on medium MS with 3.0 mg L^{-1} 2, 4-D with cotyledons as explants were 74%. The above medium of formulation was adopted for subculture; (2) the highest rates of callus differentiation medium MS with 3.0 mg L^{-1} 6-BA with cotyledons as explants was 66%; (3) the optimum medium of shoot growth was MS medium plus 1.0 mg L^{-1} 6-BA, 0.5 mg L^{-1} NAA and 0.4 mg L^{-1} GA; (4) the rooting rate was 60% on optimum rooting medium with 0.2 mg L^{-1} NAA; (5) the survival rate of plant transplanting was 70% after transferred to pots with garden soil. In conclusion, the efficient plant regeneration system developed here will be helpful for rapid micropropagation and further genetic improvement in *S. guianensis* Sw. cv. 'Reyan 2'.

Key words: *Stylosanthes guianensis* Sw. cv. Reyan 2, callus, cotyledons, plant regeneration.

INTRODUCTION

The genus *Stylosanthes* is the tropical legume, its species is variable, usually perennial, shrubby, and highly persistent. It is used as a pasture legume throughout the tropics in South America, the Caribbean, Asia, Africa, and Northern Australia (Burt and Miller, 1975). It is adapted to poor soils and a long dry season, and provides high quality forage at the end of the dry season (Humphreys, 1980). At present, there are many species, such as *S. guianensis* Sw., *S. hamata* (L.) Taub., *S. scabra* Vog., *S. humilis* Kunth, and *S. seabrana* B. L. Maass and 'tMannetje, which are widely cultivated. *S. guianensis* Sw., which is widely distributed, is cultivated in the largest area in the world with the earliest origins and richest genetic diversity of the genus *Stylosanthes* (Tang et al., 2009). *S. guianensis* Sw. cv. 'Reyan 2', one of the varieties of *S. guianensis*, is a new perennial tropical forage specie and has commonly been planted in many areas of South China (Liu et al., 1999; Stappen et al., 1999).

The *in vitro* micro-propagation offers a low-cost highly efficient technique for propagating at rates much higher than those obtained with other methods of propagation (Durako et al., 1993). So far, a series of economically important plants have been propagated through tissue culture techniques. Previous plants regenerations of the genus *Stylosanthes* were described, in which, *in vitro* regeneration of 25 species have been reported (Flick et al., 1984) in many cases at low frequencies. The *in vitro* micro-propagation in the three species of the genus *Stylosanthes* were regenerated including *S. hamata* (Scowcroft and Adamson, 1976), *S. humilis* (Meijer,

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1982a), and *S. guianensis* (Meijer and Broughton, 1981; Mroginski and Kartha, 1981; Meijer, 1982b). Plants regeneration has been obtained from protoplasts isolated from suspension cultured cells (Meijer and Steinbiss, 1983; Szabados and Roca, 1986) and leaf mesophyll tissue (Szabados and Roca, 1986). *S. guianensis* Sw. cv. 'Reyan 2' has strong drought resistance, high productivity, and high quality as a feed and long-term utilization (Liu et al., 1999), but it is susceptible to attack by chilling injury. Due to its facultative apodictic nature of reproduction, genetic improvement by conventional breeding methods is difficult. Selection of somaclonal variants in tissue culture and genetic transformation for desirable traits may be used as alternative methods to improve this species. Development of an efficient plant regeneration system for this grass is essential for genetic manipulation via somaclonal variation or transformation (Ke and Lee, 1996). The aspects of culture of *Stylosanthes* tissue and protoplast were also reviewed by Meijer and Szabados (1990). However, the study of the cotyledons-derived callus regeneration system of *S. guianensis* Sw. cv. 'Reyan 2' has not been previously reported.

The objective of this research was to build a regeneration system which might provide a good experimental foundation for genetic improvement through *in vitro* mutagenesis and other breeding programs, with the cotyledons of *S. guianensis* Sw. cv. 'Reyan 2' as explant.

MATERIALS AND METHODS

Plant material

A commercial variety of *S. guianensis* Sw., namely 'Reyan 2', was used in this study. Seed of the variety was harvested in Danzhou, Hainan, China and was kindly supplied by H.F. Huan, Tropical Crops Genetic Resources Institute, Chinese Academy Tropical Agricultural Sciences at Danzhou, Hainan, China. The seeds were stored in the dark at 4℃ until used. The cotyledons of the 'Reyan 2' were used *in vitro* for callus induction and plant regeneration.

Material sterilization and preparation

The seeds of *S. guianensis* Sw. cv. 'Reyan 2' were soaked in 80℃ water for 2 min, followed by rinsing with flowing water for 40 min. The surface of seeds was sterilized sequentially in 75.0% (v/v) ethanol for 50 s and 0.1% $HgCl_2$ for 15 min, followed by a 5 times sterilization in distilled water. When the seeds were placed on MS medium with 1.0 mg L^{-1} 6-BA and 0.7 mg L^{-1} NAA and were cultured for 15 days, seedling was 3 to 5 cm in length. Cotyledons of 1.0 cm in length and cotyledons cutting from the seedlings would be placed.

Calluses induction and subculture

MS medium was taken as the basic culture medium. Four different media were formulated for calluses induction from seeds, hypocotyls and cotyledons of *Stylosanthes guianensi*. The MS (Murashige and Skoog, 1962) medium has been widely used in previous trials (Kerry and Jordan, 2001; Chengalrayan et al., 2005; Wang et al., 2002). The MS was supplemented with a range of (1.0, 2.0, 3.0, and 4.0 mg L^{-1}) 2, 4-D (2, 4-Dichlorophenoxyacetic acid) in plastic culture bottles (240 ml, 10 cm in height and 7 cm in diameter) containing 33 ml of the medium. The culture media containing 3.0% sucrose and solidified with 0.75% agar was prepared. For calluses induction and subculture, cultures were incubated for 34 days at 25±1℃ under fluorescent light (100 µmol m^{-2} s^{-1}) in a lighting regime of 12:12 h (light/dark) at 25±1℃, to observe the effect of 2, 4-D on calluses subculture.

Shoot regeneration from calluses

To induce shoot, the callus were transferred to MS medium containing (1.0, 2.0, 3.0, and 4.0 mg L^{-1}) 6-BA under fluorescent light (100 µmol m^{-2} s^{-1}) in a lighting regime of 12:12 h (light/dark) at 25±1℃ for 28 d, and subcultured once, with the same conditions aforementioned, to observe the effect of 6-BA on shoot regeneration from calluses.

Shoot growth

The shoots were transferred and cultured for 28 days on MS medium of shoot growth with 1.0 mgl^{-1} 6-BA, (0.1, 0.3, 0.5 0.7, and 0.9 mgl^{-1}) NAA (α-Naphthyiacetic acid), and (0.4 and 0.6 mg L^{-1}) GA under fluorescent light (100 µmol m^{-2} s^{-1}) in a lighting regime of 12:12 h (light/dark) at 25±1℃ for 28 days, to observe the effect of 6-BA, NAA, and GA on shoot growth, the optimum medium for shoot growth was chosen by 2 subcultures and 28 days for each subculture.

Shoot rooting

Regenerated shoots measuring 4.0 to 4.5 cm in length were transferred for rooting medium, which consisted of quarter-strength MS medium supplemented with different concentrations of NAA (0, 0.1, 0.2, 0.3, and 0.4 mg L^{-1}) for 17 days under fluorescent light (100 µmol m^{-2} s^{-1}) in a lighting regime of 12:12 h (light/dark) at 25±1℃. For all *in vitro* studies, the pH of the medium was adjusted to 6.5 prior to the addition of agar; 33 ml medium was dispensed in glass growth vessels and autoclaved at 121℃ and 1.0 kg m^{-2} for 20 min.

Transplanting

Plantlets with roots were transplanted to the plastic pots (6.0 cm in diameter) containing autoclaved garden soil in glasshouse at 25± 1℃ , 80 to 85% relative humidity and under natural light. After 2 weeks, plants were transplanted to the clay pots containing autoclaved garden soil under the natural light and cultured for 34 days, and the numbers of the survival transplanted plantlets were recorded. Plants grew normally and attained maturity.

Statistical analysis

For induction of calluses, 40 explants were used for each treatment with 4 replications. For shoot regeneration from calluses, 80 replicates of the respective callus masses were inoculated onto the differentiation medium. 20 replicates were used for the shoots growth with 4 replications. For rooting of shoots, 40 replicates were maintained with 4 replications. 30 plantlets were transplanted to basin with soil for the survival rate of transplanted plantlets with 4 replications. The experiment was done thrice. The results were analyzed using SAS 9.0 software. Analysis of variance was calculated, significance of differences between means was

Table 1. Effect of 2, 4-D on callus induction of dormant bud (34th d).

Combination of 2, 4-D (mg L^{-1})	Primary Time of callus induction (d)	Rate of callus induction(%)	Color and state of callus (%)
1.0	8 [a]	68±2.33[c]	yellow granule
2.0	8 [a]	71±1.71[b]	yellow granule
3.0	7 [b]	74±2.04[a]	yellow granule
4.0	7 [b]	70±2.97[b]	yellow granule

Values (% ±S.D.) with column followed by the different letters are significantly different at P < 0.05.

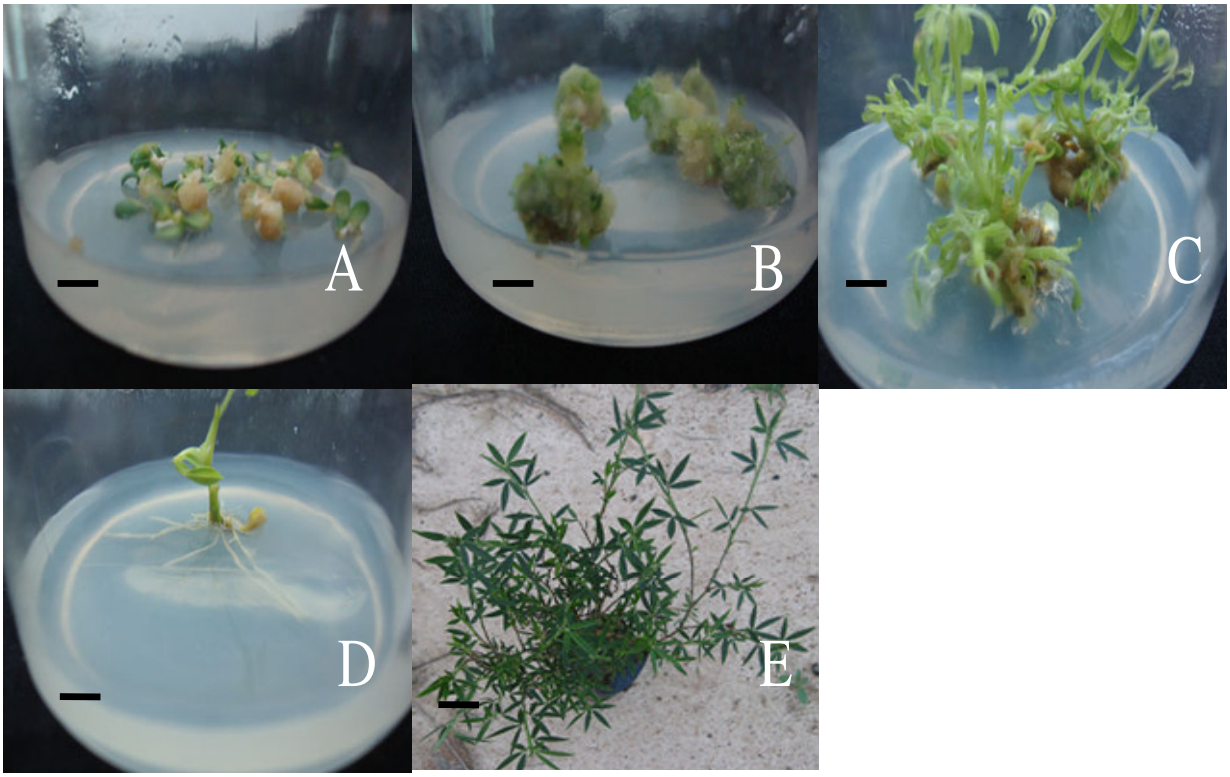

Figure 1. The formation process of regeneration plant from dormant bud-derived calluses. (A) Calluses induced from cotyledons (bar 0.5 cm); (B) shoot regeneration from calluses from seed (bar 0.5 cm); (C) shoot growth (bar 0.5 cm); (D) plantlets rooting (bar 1.0 cm); (E) transplanting survival plants (bar 4.0 cm).

conducted using Duncan's multiple-range test (Duncan 1955).

RESULTS

Calluses induction and subculture

Callus induction frequency (%) and effect in response to different 2, 4-D concentration in the MS media is presented in Table 1 and Figure 1A, respectively. Calluses formation was initiated after 13 to 15 days culture, calluses were developed in all concentration; however, callus induction frequencies differed significantly in different 2, 4-D (1.0 ≤ 2, 4-D ≤ 4.0 mg L^{-1}) concentrations. The rate of calluses induction were higher than other concentrations, and get up to 74% on the MS medium with 3.0 mgl^{-1} 2, 4-D after 34 days, color of callus was yellow granule and seedling normally grew without root. Therefore, MS based medium with 3.0 mgl^{-1} 2, 4-D was used for callus induction in subsequent experiments.

Shoot regeneration from calluses

To induce shoot regeneration from calluses of *S. guianensis* Sw. cv. 'Reyan No. 2' (Figure 1B), different BA concentrations (1.0, 2.0, 3.0, and 4.0 mg L^{-1}) with successive culture were investigated in this study (Table 2). The result demonstrated that the surface of yellow

Table 2. Effect of 6-BA on callus differentiation (56th d).

Combination of 6-BA (mg L^{-1})	Rate of shoot differentiation from yellow callus (%)
1.0	18±0.52[d]
2.0	48±2.52[c]
3.0	66±2.90[a]
4.0	54±3.14[b]

Values (% ±S.D.) with column followed by the different letters are significantly different at $P < 0.05$.

Table 3. Effect of NAA, GA, and6-BA on plantlet growth (28th d).

Combination of hormone (mg L^{-1})			Length in plantlet (cm)	Diameter of bud (mm)	Number of plantlet/every callus
NAA	GA	6-BA			
0.1	0.4	1.0	1.6±0.05[f]	0.7±0.01[b]	2.1±0.05[d]
0.3	0.4	1.0	1.5±0.15[f]	0.8±0.03[ab]	2.2±0.08[d]
0.5	0.4	1.0	4.8±0.13[a]	0.9±0.04[a]	5.2±0.11[a]
0.7	0.4	1.0	4.3±0.16[b]	0.9±0.01[a]	4.3±0.11[b]
0.9	0.4	1.0	2.1±0.12[e]	0.6±0.01[b]	4.2±0.09[b]
0.1	0.6	1.0	3.8±0.12[d]	0.8±0.02[ab]	4.4±0.10[b]
0.3	0.6	1.0	4.0±0.07[c]	0.9±0.03[a]	5.1±0.07[a]
0.5	0.6	1.0	3.9±0.06[cd]	0.5±0.01[c]	4.1±0.05[bc]
0.7	0.6	1.0	4.0±0.11[c]	0.6±0.01[b]	4.0±0.10[c]
0.9	0.6	1.0	4.1±0.08[c]	0.8±0.02[ab]	4.3±0.12[b]

Values (% ±S.D.) with column followed by the different letters are significantly different at $P < 0.05$.

granule from cotyledons became green and then produced a lot of tubers on the media with 6-BA, there was a significant correlation with 6-BA concentrations and the differentiation response. Higher concentration of (4.0 mg L^{-1}) 6-BA had no positive effect on shoot regeneration from calluses, while lower concentration of (1.0 mg L^{-1}) 6-BA could reduce the rate of shoot regeneration from calluses. The optimum media for shoot regeneration from calluses was considered as MS plus 3.0 mg L^{-1} 6-BA (Figure 1B). The highest rate of shoot regeneration gets to 66% on the MS medium with 3.0 mg L^{-1} 6-BA after culture for 28 days and once in subculture for 28 days with yellow granule callus from cotyledons. The rate of shoot regeneration from light yellow calluses was 20.0% after three subcultures, while the white sticky calluses could not generate shoots on the differentiation medium, and calluses continuously grew.

Shoot growth

The shoot growth from shoot regeneration tissues were used to test the efficiency of proliferation on different hormonal combinations (6-BA, NAA, and GA). The combination of 1.0 mg L^{-1} 6-BA, 0.5 mg L^{-1} NAA, and 0.4 mg L^{-1} GA in the MS medium, was proved best for all of the combinations which, not only the speed of plant growth would be fastest, diameter of plantlet was maximum, but also proliferation of small shoots (Table 3, Figure 1C), The media was demonstrated to be the best media formulation for shoot growth.

Shoot rooting and transplanting

Rooting percentage of shoots averaged 50% on all rooting media except on no auxins media, but there were marked differences rooting frequencies (Table 4). According to rooting quantities and diameters, the optimum rooting medium was identified to be MS + 0.2 mgl^{-1} NAA. Roots began to grow on the 8th day. After 17 days, roots developed to 1.4 cm in length, 1.0 mm in diameter and four or five in number per shoots and rate of rooting get to 60% (Figure 1D). Plantlets with roots were transplanted to the plastic pots, and then to the clay pots (Figure 1E). The survival rate of transplanting plantlets

Table 4. Effect of NAA on rooting (17th d).

Concentration of NAA (mg L^{-1})	Primary time of callus formation (d)	Root diameter (mm)	Root length (cm)	Number of root	Rate of rooting (%)
0	0	0	0	0	0
0.1	10^{a}	0.7 ± 0.07^{c}	1.0 ± 0.03^{c}	3.1 ± 0.02^{c}	41 ± 1.75^{c}
0.2	8^{c}	1.2 ± 0.08^{a}	1.5 ± 0.04^{a}	4.3 ± 0.05^{a}	60 ± 1.91^{a}
0.3	9^{b}	1.0 ± 0.05^{b}	1.3 ± 0.05^{b}	3.7 ± 0.07^{b}	56 ± 1.55^{b}
0.4	9^{b}	0.8 ± 0.03^{c}	1.2 ± 0.03^{b}	2.9 ± 0.06^{c}	43 ± 1.35^{c}

Values (% ±S.D.) with column followed by the different letters are significantly different at $P<0.05$

was 70%.

DISCUSSION

The success of *in vitro* techniques largely depends on the availability of efficient and robust tissue culture protocols. In addition, the selection of plant growth regulators and their proper combination is necessary to get high percentage of callus induction and shoot formation (Xu et al., 2009). Different explants have been used in tissue culture of forage grasses; however, the choice of explants seems largely a personal preference or based on research in cereal crops (Wang et al., 2003). In this study, protocol was established for callus induction and plant regeneration from the cotyledons calluses. Because mutagenesis and genetic transformation can easily be performed in the callus step for getting the real mutant or transgenic plants, the production of callus could be advantageous for genetic engineering and fast development of new varieties in the tropical forage legume *S. guianensis*.

In the optimum medium for callus induction, 2,4-D concentrations were identified to be 3.0 mg L^{-1} with cotyledons as explants. The optimum auxin concentrations of callus induction from leaf, which was reported by Godwin et al. (1987), were 0.5 to 2.0 mg L^{-1} 2, 4-D and 1.0-2.0 mg L^{-1} BAP. This might be caused by different genotype and explants, the high frequency of callus induction gets to 74% in our study (data not shown).

In previous research, the frequency of shoot regeneration was 59.6 % using NAA with BAP at 0.1 and 0.4 mg L^{-1} (Vieira et al., 1990) or MS + 2.0 mg L^{-1} BAP (Godwin et al., 1987). The effects of different 6-BA concentrations on shoot regeneration from cotyledons calluses of *S. guianensis* Sw. cv. 'Reyan 2' was not reported, However, many other pasture grasses has been reported, such as zoysiagrass (*Zoysia japonica*) (Lee et al., 2004) and bermudagrass (*Cynodon dactylon* L. Pers.) (Zang et al., 2006). In this study, 6-BA concentrations was 3.0 mgl^{-1} on the optimum medium of shoot regeneration from calluses from cotyledons and the percentage of shoot regeneration were 66%. Rooting was obtained in hormone-free MS medium (Godwin et al., 1987; Vieira et al., 1990). However, the percentage of rooting was only 11% (Vieira et al., 1990). To evaluate the effect of NAA on shoot rooting, NAA was applied as a rooting medium. NAA had been demonstrated as the most suitable auxin for rooting in a number of plant species in several *in vitro* trails (Maliti et al., 2005; Yuan et al., 2009; Shan et al., 2010). In this study, the highest rooting rate gets to 74%, while the optimum medium (MS + 0.2 mgl^{-1} NAA) is demonstrated to have significantly better performance than that of hormone-free MS medium.

In conclusion, this is the first report of successful callus proliferation and plant regeneration from the cotyledons-derived calluses induced from *S. guianensis*, an important economic tropical forage. An efficient plant regeneration system was established with the cotyledons-derived calluses of 'Reyan 2'. Compared with other results (Godwin et al., 1987; Vieira et al., 1990), the high rates of shoot regeneration from calluses, rooting and transplanting survival were achieved.

In this study, through the cotyledons-derived calluses of *S. guianensis* Sw. cv. 'Reyan 2', an higher efficient plant regeneration system has been successfully established, of which the rates of callus induction, shoot regeneration, rooting, and transplanting survival were 74, 66, 60, and 70%, respectively. But the rate of rooting was lower. How to improve the rate of rooting was the problem needed to be solved in the future. The creation of the callus regeneration system of *S. guianensis* Sw. cv. 'Reyan 2' provides a new way to the selection and cultivation of *S. guianensis* Sw. cv. 'Reyan 2' and it also made a sound base for its genetic transformation and somaclonal variant selection.

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