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An Effective Protocol for Callus Induction with Milk Clotting Activity from *Solanum dubium* Seeds

(Protokol Berkesan bagi Induksi Kalus dengan Aktiviti Pembekuan Susu daripada Biji Benih *Solanum dubium*)

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ABSTRACT

The aim of this study was to establish an effective protocol for callus induction from the seed explants of Solanum dubium *and to investigate the callus extract ability in milk clotting activity. The effects of growth regulator, basal media strength and sucrose were studied using different concentrations (0.5, 1.0, 1.5, and 2.0 mg/L) of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy-acetic acid (2,4-D) alone or in combination with 0.5 mg/L of 6-benzylamino purine (BAP). For milk clotting activity, about 50 or 100 µL extracts of seed callus was mixed with 2 mL 50% milk held at 55°C for 5 and 10 min until milk clotting occurred. The results showed that NAA alone or in combination with BAP gave a higher callusing percentage (80 to100%) compared to the other plant growth regulators at the same concentrations. When an auxin was supplied in combination with BAP, a significant increase in callusing percentage or degree of callusing was observed. The time required for callus to be developed was shortened and the quality of the induced callus improved. An increase in callus growth in low sucrose (10 g/L) concentration was found be comparable (88%) to high sucrose concentration (30 g/L; 60%). Crude extracts obtained from* S. dubium *callus were shown to exhibit milk clotting activity.*

Keywords: Auxin; callus; Gubbien; milk clotting activity; Solanum dubium

ABSTRAK

Kajian ini dijalankan untuk mendapatkan protokol yang berkesan bagi penginduksian kalus daripada eksplan biji benih Solanum dubium *dan untuk mengkaji keupayaan ekstrak kalus dalam aktiviti pembekuan susu. Kesan pengawalatur pertumbuhan, medium asas, kekuatan medium dan kepekatan sukrosa telah dikaji menggunakan kepekatan pengawal atur pertumbuhan (0.5, 1.0, 1.5, dan 2.0 mg/L) asid indol-3-asetik (IAA), asid indol-3-butirik (IBA), asid naftalena asetik (NAA) dan asid 2,4-diklorofenoksi–asetik (2,4-D) sendiri atau berkombinasi dengan 0.5 mg/L 6-benzilamino purina (BAP) digunakan. Untuk pembekuan susu, 50 atau 100 µL ekstrak kalus biji benih dicampur dengan 2 mL 50% susu pada 55°C untuk 5 dan 10 min sehingga pembekuan susu berlaku. Hasil menunjukkan NAA sendiri atau bersama dengan BAP memberi peratus pembentukan kalus yang lebih tinggi berbanding pengawal atur pertumbuhan lain pada kepekatan sama. Apabila auksin diberi bersama BAP, pertambahan yang signifikan dilihat dalam peratus atau tahap pembentukan kalus. Masa untuk pembentukan kalus telah disingkatkan dan kualiti kalus didapati adalah lebih baik. Peningkatan pembentukan kalus (88%) yang dicerap pada medium yang mengandungi kepekatan sukrosa yang rendah (10 g/L) didapati setanding dengan medium yang mengandungi sukrosa lebih tinggi (30 g/L; 60%). Ekstrak kasar daripada kalus* S. dubium *didapati menunjukkan aktiviti pembekuan susu.*

Kata kunci: Aktiviti pembekuan susu; auksin; Gubbien; kalus; Solanum dubium

INTRODUCTION

Plant source for milk clotting enzymes had been identified from different plants including *Solanum dubium*. *Solanum dubium* is a well-known species belonging to the family Solanaceae (Suleiman et al. 1988). It is also described as a bushy pubescent herb plant distributed in different regions in Sudan. The *S. dubium* fruit was found to have a high concentration of rennin-like compound (El-Khair & Salih 1982) that has been used for white cheese production purposes. Dawla (2001) found that cheese produced using rennin extracted from *S. dubium* is characterized by its light, soft and compact texture, preferentially better than that produced using commercial rennin materials. Recently, much research interest has been directed towards discovering a milk-clotting enzyme from natural plants that could satisfactorily replace commercial rennet in cheese making (Isam et al. 2009). In general, callus (unorganized aggregate of cells) can be induced from an excised part of a plant (explants) in which callus growth after culturing of the explants can be continued and regeneration of whole plants (plantlets) can be readily obtainable.

Therefore, the work reported here focused on the establishment of callusing from *S. dubium* seeds and to investigate the properties of the callus in milk clotting activity. The effects of various concentrations of naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or 2, 4-dichlorophenoxy–acetic acid (2, 4-D) alone (Figure 1) or in combination with 6-benzylamino purine (BAP) (Figure 2) were observed.

Materials and Methods

Plant materials

S. dubium seeds were obtained from Abul-gassim farm, Khartoum state (Sudan), and were used as source of explants throughout this experiment.

Sterilization of explants

The hard covering of the seeds was removed and the seeds were then washed and cleaned carefully under running tap water for 60 min to remove all the phenolic acid compounds. Next, the seeds were allowed to soak in a 70% (v/v) ethanol for one min. Then the seeds were soaked in 30% (v/v) Clorox with two drops of Tween-20 for 10 min followed by rinsing three times with sterile distilled water. Finally, the seeds were soaked in 1000 ppm gibberellic acid (GA_3) (Jingkai et al. 2005) for two hours at room temperature followed by rinsing three times with sterile distilled water.

Media preparation

The medium used for callus culture was Murashige and Skoog (1962) (MS), supplemented with 30 g/L sucrose, 100 mg/L myoinstol, 0.4 mg/L thiamine-HCl and 8 g/L agar. All media used in this experiment were adjusted to pH 5.8, and then distributed into 25 × 150 mm^2 culture tubes, 15 mL each and were covered with plastic Bellco kaptus before autoclaving at 121°C for 15 min under pressure of 15 psi. The media were left to cool slanting in the culture room until use.

Inoculation of explants

Under laminar air flow, the seeds were cultured on MS media then placed in an incubator at 25°C with light of 22.26 × 10^{-2} μEcm^{-2}s^{-1} for 16 h photoperiod using fluorescent lamps. The resulting callus was transferred to fresh media every four weeks.

Effects of growth regulators on callus growth

This experiment was designed with four different concentrations (0.5, 1.0, 1.5 and 2.0 mg/L) of IAA, IBA, NAA and 2, 4-D alone or in combination with 0.5 mg/L of BAP. MS basal medium (MS macro + MS micro) supplemented with 8 g/L agar was used throughout the experiment. All treatment combinations were replicated at least 15 times, and data for callusing percentage and degree of callusing were recorded after eight weeks of culture.

Effects of sucrose and basal medium strength on callus growth

Effects of sucrose and basal medium strength on callus growth experiment was designed with sucrose concentrations of 10, 20 and 30 g/L in half and full strength MS basal medium with 1.0 mg/L of NAA in combination with 0.5 mg/L of BAP.

Extraction of enzymes

The freeze-dried powdered callus (10 g) *S. dubium* were suspended in 100 mL of 50 mL phosphate buffer pH 7.1 containing 0.01% (v/v) sodium azide, gently stirred for 24 hours at 4°C. The crude callusing extract, obtained after filtration and centrifugation at 10,000 rpm for 15 min, was stored at 4°C, while the residues were discarded.

Milk clotting activity of crude extrct

The supernatant of crude callus extract (50 or 100 μL) was mixed with 2 mL of 50% milk (fresh milk diluted in sterile distilled water pH 5.4) at 55°C until milk clotting occurred. One unit (1U) was defined as being the quantity (ml) of crude protein extract needed to coagulate 2 mL of 50% milk in 5 min at 55C°. This method was found to be suitable for milk clotting (Arima & Iwasaki 1970). The milk clotting unit is defined as the amount of enzyme that clots 10 mL of the substrate within 40 min and the milk clotting activity (*MCA*) can be calculated as in the following equation:

$$MCA(U/mL) = \frac{2400}{clotting\ time \times dilution\ factor} \quad (1)$$

Results and Discussion

The results showed the effect of four types of auxin treatments on callus formation in *S. dubium* seeds. The results also indicated that the S. *dubium* callus growth was remarkably affected by the growth regulator type and concentration. While there was a little expansion of the explants, no callus growth was observed during the culture period in the control.

Callusing percentage in seed culture of *S. dubium* was highest when treated with auxin at 0.5 mg/L concentration, and decreased with higher concentrations of auxin (Figure 1). These results showed that seeds were enlarged and developed callus at the cut surface in all media within one to six weeks of inoculation depending on auxin type and concentration. Of the various auxins tested, NAA at different concentrations induced rapid, highest callusing percentage, healthiest, invariably greener and more granular callus from seed explants compared to IAA, IBA and 2, 4-D when supplied at the same concentrations (Figure 1).

The effects of auxin at different concentrations in combination with BAP induced significant increase in callusing percentage or degree of callusing when compared to auxin alone (Figures 1 and 2). Furthermore, the time required for callus to be developed on the surface of seed explants was shortened to two weeks. The appearance of the induced callus was improved, became more viable and looked healthier even at higher auxin concentrations. The results also showed that auxins at 0.5 mg/L concentration with BAP promoted rapid high callusing percentages (Figure 2).

Increasing sucrose concentration in the basal media showed decreasing callus induction regardless of the MS basal medium strength (Figure 3). Nevertheless, basal media supplied with 10g/L sucrose showed distinctly better callusing percentages than with 20 or 30g/L sucrose in both half and full MS basal medium strengths. Furthermore, in agreement with our results, neem was found to show callus induction at low sucrose concentration compared to high concentration (Wewetzer 1998).

Callusing response and callus appearance were better in full strength MS basal medium than half strength for

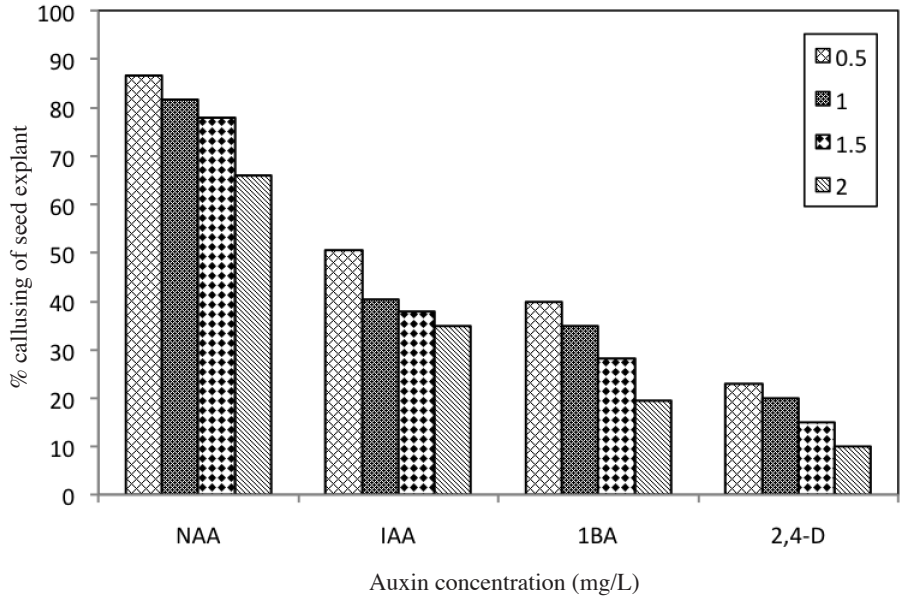

FIGURE 1. Callusing percentage of *S. dubium* seeds cultured on MS medium with different auxins at various concentrations after 6 weeks of culture

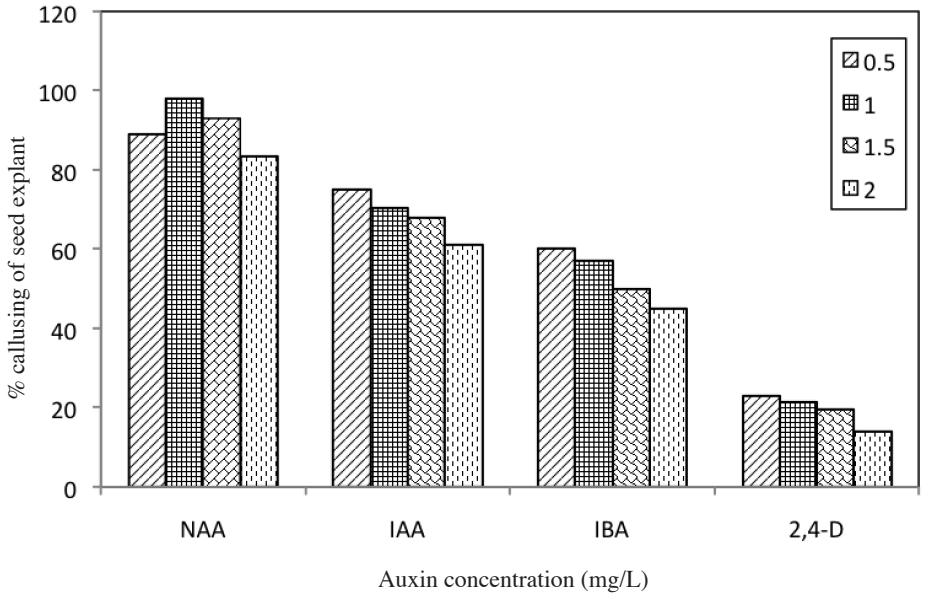

FIGURE 2. Callusing percentage of *S. dubium* seeds cultured on MS medium with different auxins at various concentrations with 0.5 mg/L BAP after 6 weeks of culture

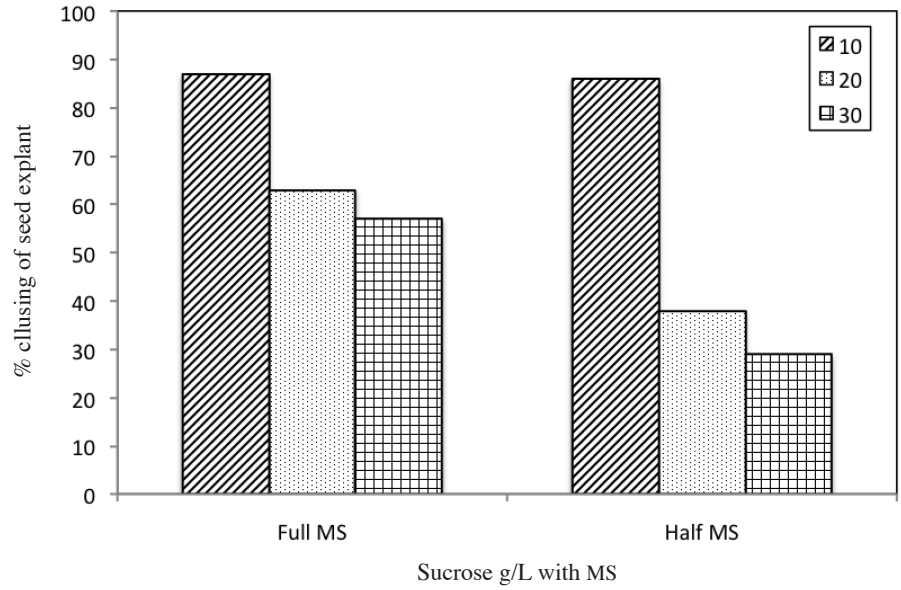

FIGURE 3. Callusing percentage of *S. dubium* seeds cultured on MS medium with different concentrations of sucrose after 6 weeks of culture

all levels of sucrose concentrations tested. The culture conditions for *S. dubium* seed callus induction and growth were established. We have shown that the presence of auxin alone or in combination with cytokinin in particular BAP in the medium was capable of inducing callus. However, the callusing percentage, degree of callusing and callus appearance were dependent on auxin type and concentration. The seed explants induced the best callus when cultured on full strength MS basal medium supplemented with 1.0 mg/L of NAA, 0.5 mg/L of BAP and 10 g/L of sucrose. However, it failed to produce callus in media without auxin, emphasizing that *in vitro* callus formation is attributed to the presence of growth regulators in the medium. Generally cytokinin and auxin are known to promote callus formation in tissue culture (Akiyoshi et al. 1983; Letham 1974; Skoog & Armstrong 1970). Moreover, similar findings have been observed for neem callus induction where IBA was used in combination with BAP (Murthy & Saxena 1998).

Comparison of the milk-clotting activity among various coagulants showed that *S. dubium* callus gave highest clotting activity (Table 1) at 900 units/ mL whereas rennet showed lowest activity at 249.6 unit /mL. Thus *S. dubium* was more active in coagulation compared to rennet. The crude callus extracts with and without calcium coagulated the fresh milk better than the control sample (Figure 4).

CONCLUSIONS

The *S. dubium* callus growth was remarkably affected by growth regulator type and concentration. Callus growth was not observed during the culture period in the control (basal medium without growth regulator) even though there was a little expansion in the growth of the explants. The presence of auxin alone or in combination with BAP in the medium was capable of inducing callus. However, the callusing percentage and the degree of callusing depended on auxin treatment. The *S. dubium* induced the best callus when cultured on full strength MS basal medium supplemented with 1.0 mg/L of NAA, 0.5 mg/L of BAP and 10 g/L of sucrose. In addition, the extracts derived from *S. dubium* callus have demonstrable milk clotting activities and also exhibited that *S. dubium* callus production from seeds may be a promising source for low cost enzyme with milk coagulation activity.

TABLE 1. Summary of coagulation materials and their clotting activity

	Clotting Activity (units/mL)
[a]Rennet from calf	249.6
[a]Mucor rennet from calf	551.0
[a]*Endothia parstica* enzymes from microbe	750.0
[a]Papain from plant	216.0
[b]*Solanum dubium* seeds from plant	880.0
[c]*Solanum dubium* callus from seed	900.0

[a] Arima et al. (1968), [b] Isam et al. (2009) and [c] the present work.

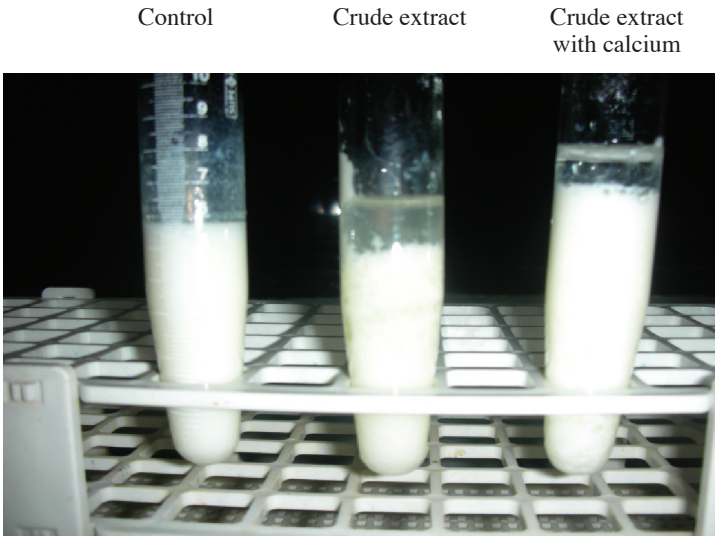

FIGURE 4. Coagulation of fresh milk by crude extract of callus from *S. dubium* seeds

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