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Regeneration of Cowpea *Vigna radiata* L. Plant from Cell Suspension

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ABSTRACT

This study dealt with the establishment of cell suspensions culture from cotyledonous stems callus of *Vigna radiata* L., using an MS medium supplemented with 1.0 mg^{-1} Naphthalene acetic acid (NAA) and 0.5 mg^{-1} Benzyl adenine (BA). Two densities of cell suspensions were grown using two types of media. The first medium, MS0, was devoid of growth regulators. The density of the third day was higher than that of the fourth day. The other medium is MS containing growth regulators, and the third-day density was also higher than the fourth-day density in culture suspensions. the cell density grown in MS medium supplemented with growth regulators is higher than the density of cells growing in MS0 medium devoid of growth regulators.

Keywords- Cowpea (*Vigna radiata* L.), Cell suspension cultures, NAA (Naphthalen acetic acid), BA (Benzyl adenine), MS (Murashige & Skoog medium).

I. INTRODUCTION

Cowpeas are a grain leguminous herbaceous plants that possess a strong root system. Root nodules contain specific symbiotic b *Rhizobia* that fix atmospheric nitrogen N_2 in the form of nitrate (Sheahan,2012). Cell suspensions are efficient systems of particular importance to the investigator in the fields of genetic engineering, cell biology, and biochemistry, as they give prospects for studying the cell life cycle with high accuracy. Also, provide a homogeneous plant material that grows and divides in tight conditions. (Santos *et al*., 2016). Many reports mentioned that cell suspension contributes to understanding the behavior of a single cell from the first day of division and the sequential divisions (Moscatiello *et al.*, 2013). The formation of cellular colonies and the interpretation of physiological activities at the cellular level. This contributed to overcoming many difficulties in the development of callus and its differentiation in economic and medical leguminous plants (Havenith *et al*., 2014). Cellular suspension cultures have invested in an alternative source to produce high-value biologically active compounds in some plant species (Satdive *et al.*, 2015). Cultivation of various densities of these suspensions by embedding in multiple agar droplets directly showed that their cells begin to divide and form cell colonies within 3 to 5 days, then develop into callus primordia, which were successfully transferred to the culture medium (Rasheed and Kassim, 2006).

This study aimed to produce Cowpea plants from cell suspension-derived callus, A/So

To defect if regeneration cowpea plants conserved their symbiote association with the specific rhizobium.

II. MATERIALS AND METHODS

- ***Hypocotyl Production***

Thirty cowpea seeds were surface sterilized by immersing in 96% ethyl alcohol, with continuous stirring for two min., then immersed in sodium hypochlorite NaOCl solution (Fas, produced by Babylon Detergent Factory / Baghdad) at a ratio of 1.0: 2 v:v of water (Al-

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Jawari, 2004) for 15 min. Seeds were removed and washed with sterile water 3 times (Arife and Muhammad, 2019). Sterilized seeds were placed on sterile filter paper, and planted on the surface of 30 ml of agar-solidified MS0 medium in 70 ml capacity test tubes (two seeds/ tube). Samples were kept in the culture room, in complete darkness, for the first three days, (25 ± 2 °C) until the emergence of root and the beginnings of the shoot. Later samples were transferred to successive light and dark conditions (16 h. light / 8 h. darkness, light intensity 2000 lux) until the formation of intact seedlings (Badrani and Al-Bakr, 2021).

Preparation of explants from axenic seedlings

Healthy cowpea seedlings of age 10 to 15 days, produced from surface sterilized seeds, were removed from the culture medium. Stems were cut into 1.0 cm lengths, and the leaf was cut into pieces of 0.5-1.0 cm^2. Both types of explants were used in callus production (Raghavendra, 2018). Each of stem and leave explant was placed on the surface of 30 ml of MS + 1.0 mg^{-1} NAA+ 0.5 mg^{-1} BA In 100 mL glass flasks. Flasks were closed with aluminum foil. Samples were kept in the culture room condition previously mentioned.

Indication of callus from stems and leaves explants.

Stems and leaves explants were placed in three pieces on the surface of 30 ml of the induction (3 explants / flask), medium in 100 ml glass flasks, and sealed with aluminum foil. The following culture mediums were tested.

MS0 (Control)
MS+ 0.5 mgl^{-1} NAA + 0.5 mgl^{-1} BA
MS + 1.0 mgl^{-1} NAA+ 0.5 mgl^{-1} BA
MS + 1.5 mgl^{-1} NAA + 1.0 mgl^{-1} BA
MS + 2.0 mgl^{-1} NAA + 1.5 mgl^{-1} BA (Al-Jawari, 2004)

Establishment of cell suspensions from callus

One gram sample of young and friable calli was placed in 100 ml glass flasks, each flask containing 50 ml of liquid medium MS + NAA 1.0 mg^{-1} and BA 0.5 mg^{-1}. flask incubated in a shaking incubator (New Brunswick, USA) in complete darkness at 25°C, 150 rpm (Morris and Fowler, 1981). After 24 hours of incubation, they sieved through a sterile plastic 46µm sieve (PGMG, UK.) to remove cell clumps. Cultures were returned to the shaking incubator under the same conditions. Later, cell suspensions were subcultured by removing the glass flasks from the incubator and placing them inside the cabinet obliquely, for 4 hours to allow the cells to precipitate, disposing of the liquid medium by pouring carefully without losing the stable cells. Adding a similar volume of the same fresh prepared medium, return to the shaking incubator (Greshhoff, 1980). Cultures were distributed in 10 ml test tubes centrifuged (Jouan BR311) at 1500 rpm for 5 min., where the cells settle. at the bottom of the tubes.

Determination of cell suspension viability

Viability of the cells of suspensions was estimated following the standard method (Birkenhead and Willmer, 1986) Evan blue stain solution (Chemical Ltd. Poole, England BDH) was prepared by dissolving 0.5 g in 100 ml of distilled water, mixing 0.1 ml of the dye solution with 0. 1 ml of cell suspension. Samples were left for 10 min. examined by a light microscope. The dead cells appeared blue, while the viable cells remained without stain. Number of viable cells was calculated. according to the following equation:

$$\textbf{Viability} = (\textbf{number of dead cells} / \textbf{total number of cells}) \times 100$$

…(Paul,1970)

Embedding of cell suspensions in agar drops

Take 1.5 ml of cell suspension culture grown in liquid medium (MS + NAA 1.0 mg^{-1} + BA 0.5 mgl^{-1}) and mix with 1.0 ml of 3% solution of previously dissolved sterile agar, placed in a water bath at 40 °C. mix carefully in a test tube. The mixture was distributed in equal drops of 0.2 ml / each in the bottom of a plastic Petri dish (9 cm diameter). Dishes were left open in the cabinet to solidify. Then, 5.0 ml of liquid medium MS+BA 0.5 mgl^{-1}+ NAA 1.0 mgl^{-1}+25g Manitol was added to each plate and not to submerge the drops completely. Dishes were covered with lids and sealed with parafilm. Dishes were kept at 25°C, light intensity of 700-800 lux, 16 hr. light / 8 hr. darkness (Bharathi and Elavarasi, 2012).

Transfer of callus primordia

Callus primordia developed in agar droplets were transferred as reached a reasonable size and were seen with the naked eye. As they emerged from agar droplets, drops were transferred completely (Mohammed and Al-Mallah, 2013) to the surface of 30 ml of agar solidifed MS + BA 0.5 mgl^{-1} + NAA 1 mgl^{-1} medium. Samples were incubated in culture room condition.

III. RESULTS

Callus production

Data showed that the friable callus was amenable to the formation of cell suspensions. Cultivation of cell suspension densities of third and fourth days divisions in completely dark conditions when they were cultured in MS medium supplemented with growth regulator and mannitol mentioned earlier. After 50 days of cultivation, callus primordia developed from cell suspensions and was seen by the naked eye, which led to the cracking of the agar. Calluses were transferred from agar droplets to differentiation media MS + NAA 1.0 mgl^{-1} + BA 0.5 mgl^{-1}. Callus derived from cell suspensions was distinguished by its fragile texture and green color.

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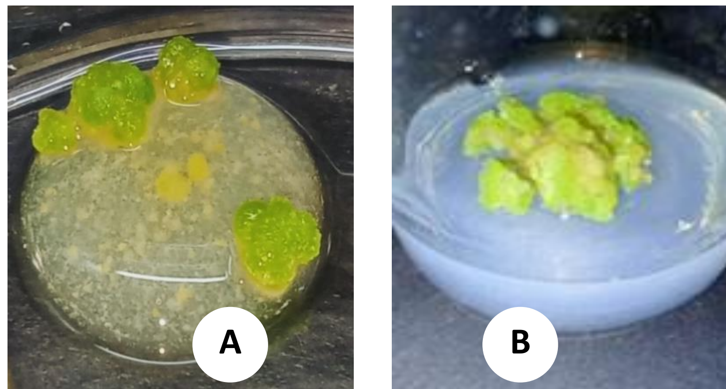

Figure 1: Callus produced from cell suspensions of cowpea plants *Vigna radiata* L.
(A) Callus produced from cell suspensions.
(B) Callus cell suspensions have grown on MS medium containing growth regulators MS+ 1.0 NAA mgl^{-1} +0.5 BA mgl^{-1}

Preparation of cell suspension

Data induct that culture of two different densities third and fourth day start to divisions in completely dark conditions as grown in MS medium supplemented with the aforementioned growth regulators MS+ NAA 1.0 mgL^{-1} + BA 0.5 mgL^{-1} and mannitol, and the other MS medium devoid of growth regulators for a callus of sub cotyledonous stems. The single cells made the first division after 3 days of cultivation produce daughter cells. The second division occurs two days after the first division . The formation of the three-celled stage and developed towards the four-celled stage, and this division took 5-8 days and continued with successive divisions until it reached the formation of cellular colonies, After 15 days, the first callus initiators appeared, represented by small pieces of callus immersed in solid agar drops for suspensions containing growth regulators. After 20 days, the first callus primordia formed an MS medium containing growth regulators. Callus primordia were carried over to the cascading light and dark conditions 16/8 until the initiators developed and transformed into callus pieces, increasing their size, and cracking the agar drops.

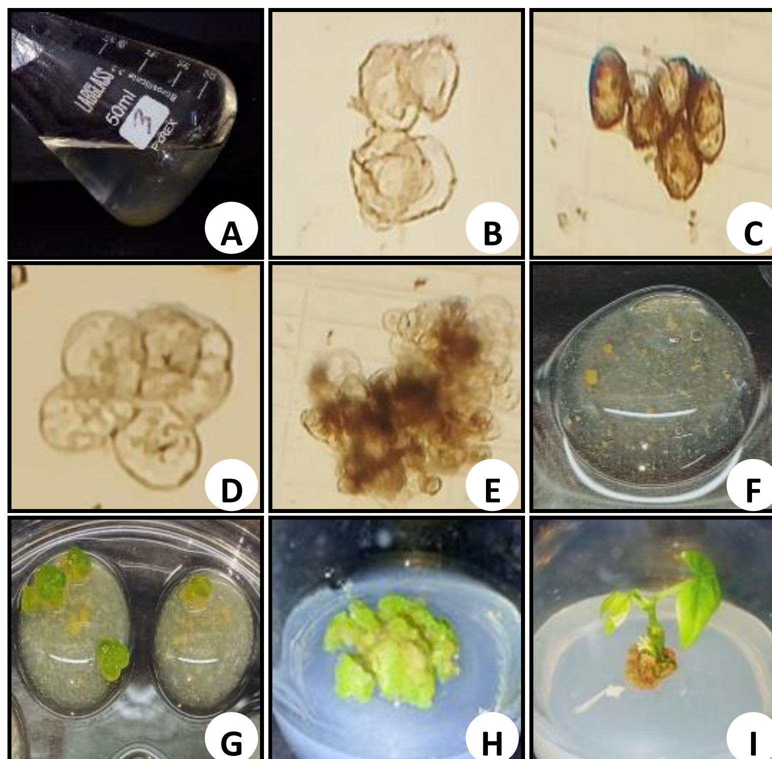

Figure 2: Stages of cowpea (*Vigna radiata* L.) callus formation from cell suspension cultures.
(A) Culture of cell suspensions derived from hypocotyls callus in liquid induction medium MS +NAA 1.0 mg l^{-1} + BA 0.5 mg l^{-1}.
(B) Mitosis of cell and the formation of two nucleated cells.

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(C) Mitosis of a cell (B) and the formation of 4-cell stags
(C,D) Formation of triple and quadruple cell stages.
(E) Initiation of the cellular colony.
(F) Cell colonies callus primordial developing.
(G) Cell suspensions cultured on MS + 1.0 NAA mgl^{-1} + 0.5 BA mgl^{-1}.
(H) Callus produce from cell suspension cultures.
(J) Cowpea plant formation from differentiation of cell suspension callus

Production of cell suspension culture

The results indicated that callus was amenable to creating numerous single cells producing homogenized fine cell suspensions. Data showed a subsequent increase in cell densities up to the third day of culture age (Table 1).

Table 1: Establishment of cell suspension from hypocotyl friable calli of *cowpea (Vigna radiata* L.) plant using two different types of media:

	Densities (×10^4 cell\ ml)				
	(Days)				
Medium mgl^{-1}	**First**	**Second**	**Third**	**Fourth**	**Fifth**
MS0 (control)	18	25	33	21	15
MS+ 1.0 NAA + 0.5 BA	22	45	50	42	30

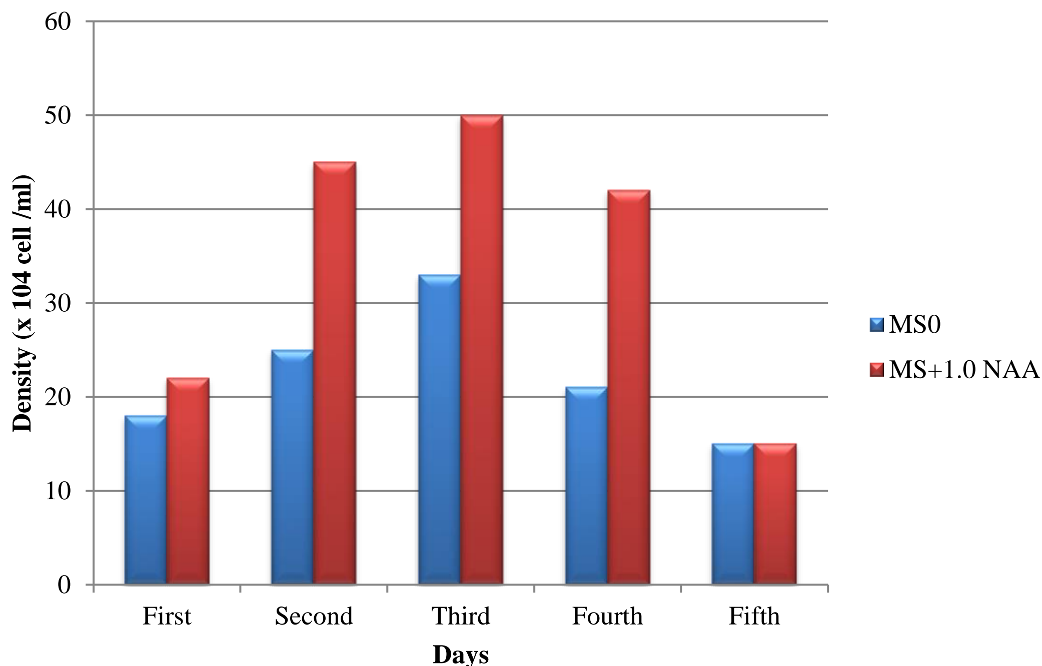

Figure 3: Cell suspension density (x 10^4 cells/ml) in culture medium MS0 and MS+ 1.0 NAA mgl^{-1} +0.5 BA mgl^{-1}

Embedding in agar

Table 2: Development of callus primordia from cell suspensions embedded in agar droplets liquid MS medium by multi-agar drops

medium (mgl^{-1})	number of drops	number of drops that create a callus	Period (Day)	Callus (%)
MS0 (Control)	7-9	6	20	30
MS+ 1.0 NAA +0.5 BA	7-9	10	15	50

Average number of drops 8-10 drops/dish

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Table 3: Cultivation of cell suspensions at day 3 and 4 density on MS0 medium from callus of cowpea plants (*Vigna radiata* L.)

Medium (mgl^{-1}) MS0	Densities $\times 10^4$ cell\ ml	Number of drops			
		Planted	Developed	Callus Primordial	Primordia
Third day	33 cell	45	22 drops	4 drops	After 20 days
Fourth day	21 cell	40	20 drops	3 drops	After 20 days

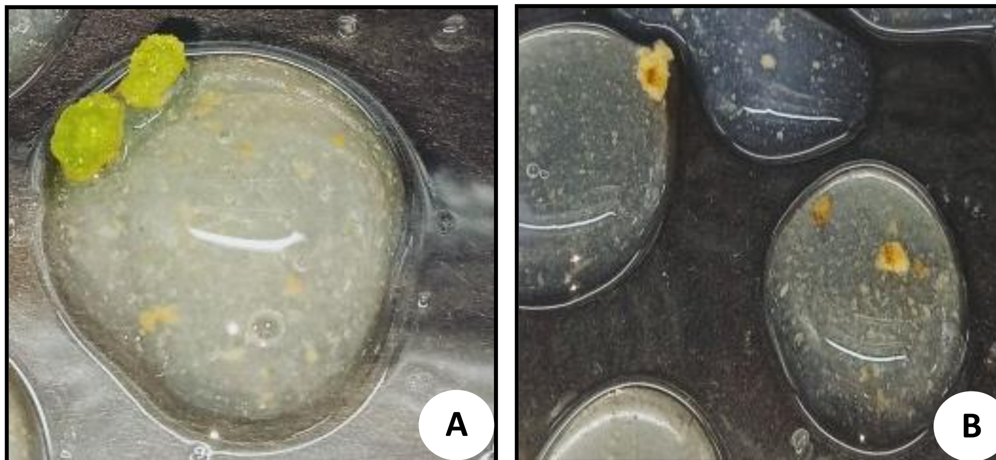

Figure 4: Cell suspensions cultured on MS0 medium of cowpea plants (*Vigna radiata* L.)
(A) Callus cellular suspensions implanted on the third day.
B) Callus cellular suspensions implanted on the fourth day.

Table 4: Cultivation of cell suspensions at the density of the third and fourth day on MS medium containing growth regulators from the callus of cowpea plant (*Vigna radiata* L.)

Medium (mgl^{-1}) MS+ 1.0 NAA +0.5 BA	Densities $\times 10^4$ cell\ml	Number of Drops			
		Planted	Developed	Callus primordia	Primordia
Third day	50 cell	35	25 drops	4 drops	After 15 days
Fourth day	42 cell	40	24 drops	4 drops	After 15 days

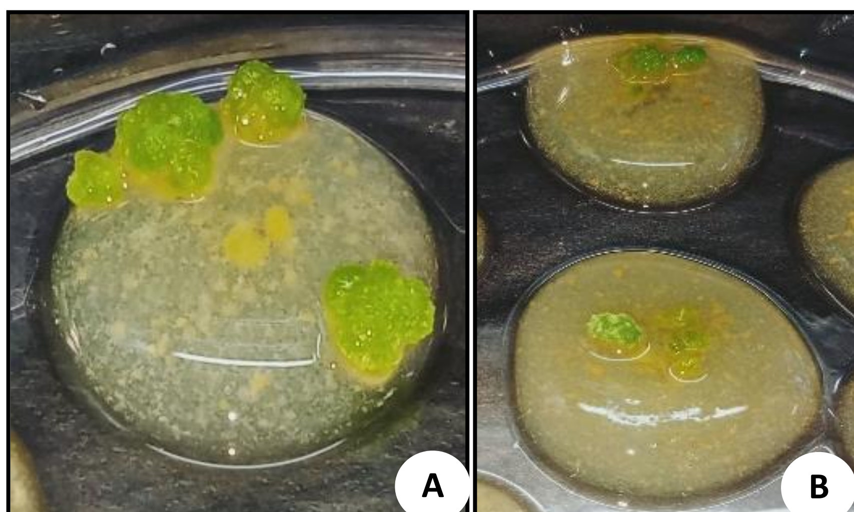

Figure 5: Cultivation of cell suspensions at the density of the third and fourth day on MS medium containing growth regulators from the callus of cowpea plant (*Vigna radiata* L.)
(A) Callus cellular suspensions implanted on the third day.
(B) Callus cellular suspensions implanted on the fourth day.

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IV. DISCUSSION

The results of the current study showed a high variability in callus formation, approximately 90-100% of the eradicated plants started nodules from Gram green callus when cultured on modified MS media containing 0.5 mg/L BA profuse callus induced regardless of the explants used, BA-free media failed to induce callus from cotyledonous nodules. It reveals that for the induction of callus from cotyledonous ganglia, cytokinins may be indispensable (Khatun *et al*., 2008). Cultivation of excised plants on MS medium initially resulted in green calluses on MS medium containing BA alone and greenish nodular callus was obtained when BA was used in combination with NAA. Callus growth was higher in the medium containing a concentration of BA along with NAA. MS media supplemented with 3.0 mg/L BA combined with 0.5 mg/L NAA produced 92% callus from plant explants of stems and leaves. MS media supplemented with 2.5 mg/L BA along with 0.5 mg/L NAA produced a better pathway with higher growth. The highest callus bud regeneration was found on MS media containing 2.5 mg/L BA (Aparna *et al*.,2018). In general, Vigna regeneration in vitro is more limited than that of other legumes. In Vigna radiata L. plants are directly regenerated from explants, for example, cotyledon tips, cotyledons, primary leaf, and hypocotyl. Regeneration of shoot buds from cotyledon tissues of Gram green was grown on MS medium supplemented with various concentrations of BA or Kinetin with NAA or IAA. No callus formation and the bud were budding without a growth regulator in the MS medium. Among the cytokines tested, chitin with BA showed a higher rate of cotyledon bud regeneration (Varalaxmi *et al.,*2007). the plant species of the legume family in the culture media can form and differentiate calli (Al-Bayati, 2002). The success of establishing cellular suspensions from the fragile callus of stems is explained by the ease of separation of its cells and its containment of large numbers of Single cells and their divisions and formation of callus facilities are likely to continue due to the abundance of essential nutritional needs in the presence of NAA (Mohammed and Al-Mallah, 2013; Neumann *et al*., 2009). Auxins and cytokinins are an essential factors for the formation of calli, not only in leguminous plants, but also in all plant species, because they have a major role in enabling cells to divide, forming unspecialized callus tissue, Auxins are responsible for increasing the softness of the cell wall and increasing its permeability, moreover, they have an effect on metabolism and Nucleic acids (Dietz *et al*., 1990). This encouraged the cells to divide and behave in the usual pattern in their successive divisions and the formation of calli. The cellular suspensions of leguminous plants, including chickpeas, are similar to their counterparts in several other plants in the legume species such as beans, cowpeas, and lentils (Hernandez-Garcia, 2021). The reason for adopting cell suspension cultures derived from callus is that the plant is produced from a single cell or groups of them (Moscatiello *et al*.,2013) It is due to the success of the MS medium in establishing cultures of cell suspensions, as indicated by the multiplicity of cells and their vitality, and that this medium contains a large number of dissolved sugars in it. (Silvereira *et al*.,2009). The process of cultivating cell suspensions by burying them in multiple agar droplets is a technique for obtaining a fragile callus and then differentiating it into a complete plant, Through this study, a fragile callus was obtained from cell suspensions and transferred to a differentiation medium to obtain vegetative branches, and the branches were rooted to obtain the whole parts cowpea plant Another study showed the ability to calcify cell suspensions grown from the production of bean plants by burying them in agar drops(Al-Jwari,2004). The cell suspensions took a normal course in their growth and led to an increase in volume and callus formation, as well as success in the differentiation process and the emergence of vegetative branches on MS medium is supported by the growth regulators BA, NAA (Binding and Nehls, 1978).

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