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# Factors Affecting Asexual Embryogenesis Via Callus in *Theobroma cacao* L.

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ABSTRACT. Asexual embryogenesis occured spontaneously in embryogenic-competent Callus of Cacao (*Theobroma cacao* L.) obtained from certain clones of asexual embryos. The Coconut water (CW) promoted embryo initiation and development at 10% and 20% w/v respectively. Cultures previously rejuvenated with coconut water (CW) developed better than non-rejuvenated and the effect was greatest in liquid than in semi solid media. The effect of other growth factors was studied.

The desire to obtain large numbers of propagules from high yielding cultivars has stimulated tissue culture studies in cacao. A second objective of the tissue culture approach has been to produce cocoa butter or cocoa flavor *in vitro* from callus (Tsai and Kinsella 1981) or from culture of axesual embryos (Janick *et al.* 1982).

Micropropagation of cacao was first suggested by Archibald (1954) who successfully induced callus from cambium tissue using White's medium plus coconut water. However, organogenesis from this callus was not obtained. Since then, several sporadic reports particularly aimed at propagating cacao *in vitro* from callus or organ cultures have been published (Hall and Coolin 1975, Esan 1977, Orchard *et al.* 1979, Pence *et al.* 1979, Kong and Rao 1981, Passey and Jones 1983, and Elhag 1985). Callus was initiated from all explants, but the only organized growth from callus was roots. Proliferation from excised shoot apices (Orchard *et al.* 1979 and Passey and Jones 1983) or from cotyledonany nodal tissue of cacao seedling (Janick and Whipkey 1985) was not succesful and have indicated the

intractability of the cacao species with respect to regeneration from callus or shoot tips.

Propagation of superior clones of cacao would require induction and subsequent development of asexual embryos from somatic tissues of mature sporophytes. In addition, somatic embryos may offer a means for the *in vitro* production of secondary metabolites normally produced in seeds if asexual embryos can be induced to develop normally.

Asexual embryogenesis in *Theobroma cacao* L. was first reported by Esan in 1977 and independently by Pence *et al.* in 1979. Certain clones of asexual embryos originally obtained from zygotic embryos as described by Pence *et al.* (1979, 1980), produced embryogenic callus spontaneously on hormone-free basal medium (Kononowicz *et al.* 1984), but embryogenesis was typically of low or variable frequency.

The purpose of this study was to investigate the effect of different factors on the induction and subsequent development of asexual embryos from embryogenic callus of cacao.

## Materials and Methods

The basal culture medium consisted (in mg/liter) of Murashige and Skoog (1962) salts, 0.1 thiamine-HCI; 0.5 pyriodoxine-HCI, 0.5 nicotinic acid; 2.0 glycine; 100 i-inosital; 1000 casein hydrolysate; 15000 sucrose and 8000 Bacto-agar. Media were sterilized by autoclaving for 15 min at 121°C after adjusting the pH to 5.7. Media were distributed as slants of 10 ml into sterile petri dishes (15×60 mm). All cultures were kept under low intensity illumination (ca. 1.5 KIx)) from cool white fluorescent lamps per 16 hr daily at 26°C. All experiments were initiated using callus clumps measuring approximately 2-4 $mm^2$ (4 pieces/plate) as explants. Callus was checked under the microscope for the presence of embryos immediately after transfer and at the end of the culture period.

### *Growth Factors*

The basal medium was supplemented with various concentrations of growth substances as described. These included 2,4-D,BA,$GA_3$, adenine sulfate, ammonium sulfate, proline and coconut water (CW). All factors were added to the basal medium before autoclaving except $GA_3$ which was filter sterilized and added to the cooled media after autoclaving.

### *Plant Material*

Budding centers and callus of clones BC5, 36 and 46 were maintained as stocks by subculturing on semisolid media with 1.5% sucrose and without growth regulators every month.

### *Growth Measurements of Callus*

Callus growth was measured by calculating the area:

$$\text{Callus area} = D_1.D_2/4$$

where $D_1$ is the largest diameter and $D_2$ the perpendicular diameter (Dale and De Ambrogio 1979).

### *Embryogenesis*

The number of cotyledonary embryos was counted at the end of each experiment. In experiments where a mass of globular embryos was produced the frequency of embryogenesis was determined by estimating, the ratio of globular embryonic tissue to callus for each clump based on area. (Fig. 1)

## Results

Experiments were initiated in semisolid media to evaluate a number of growth factors that were thought to affect embryogenesis in cacao and other species (Elhag 1985). Thus, various concentration of 2,4-D, $GA_3$, BA, CW, proline, ammonium sulfate and adenine sulfate were studied using embryogenic-competent callus of clones BC 5 and BC 36.

Results shown in Table 1 indicated different responses due to clone with BC 36 more responsive in general than BC 5. The most response in terms of number of developed embryos and frequency of embryogenesis was obtained with 20% CW in both clones. The response to proline was different in the two clones. Higher proline levels (10,100 mM) increased the embryogenic frequency in BC 5, while lower levels 0.1 and 1.0 mM increased the frequency in BC 36. Both clones responded to adenine sulfate with the maximum response at 1.0 mg/liter in BC 5 and all levels above 0.1 mg/liter in BC 36. $(NH_4)_2\ SO_4$,2,4-D, BA, and $GA_3$ inhibited embryogenesis at all levels tested in BC 5, while BC 36 responded variably to the same factors. For example, $(NH_4)_2\ SO_4$ increased the frequency of embryogenesis at all 3 levels tested, while only low levels (0.01 and 0.1 mg/liter) of 2,4-D and BA slightly increased the embryogenic frequency over the control. $GA_3$ produced little effect at any concentration tested in BC 36.

The response to CW and proline was intensified at 8 weeks in culture, but there was little change in the response to the other factors after 6 weeks (data not presented). Callus growth in BC 36 was much reduced by all factors tested at all levels except CW while BC 5 responded variably.

The effect of CW, $GA_3$ and 2,4-D was re-evaluated using embryogenic callus of three clones (BC 5, 36 and 46). CW was tested at 3 levels (0,10 and 20%, v/v).

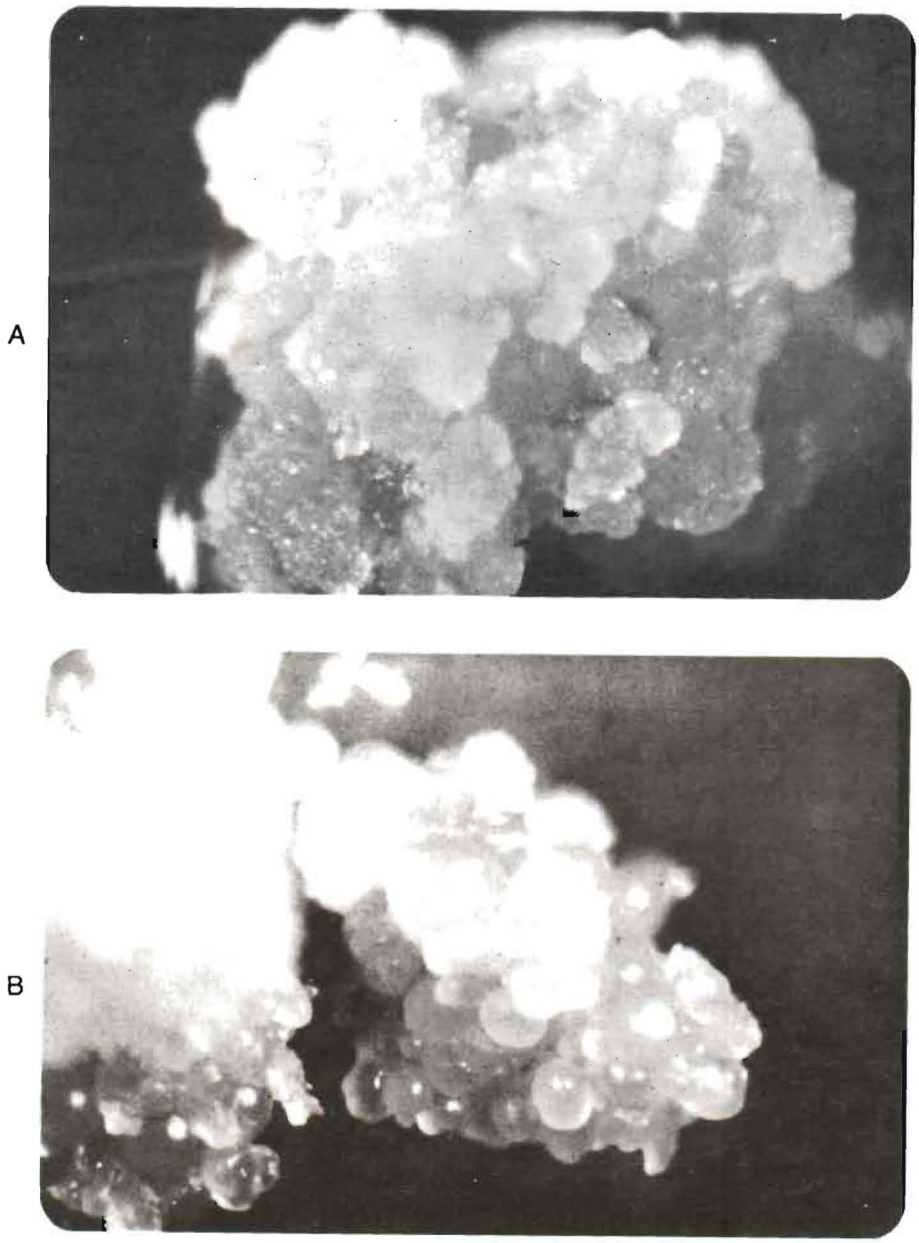


**Fig. 1. A)** Embryogenic-competent callus spontaneously produced from asexual embryos on basal medium.
**B)** Embryogenic callus turning to a mass of globular embryos upon various medium treatment.

**Table 1.** Effect of various growth factors on asexual embryogenesis in embryogenic competent callus of clones BC 5 and 36.

| Factors | Concn | BC 5 | | | BC 36 | | |
|---|---|---|---|---|---|---|---|
| | | Embryo-genesis (%) | No. cotyledonary embryos | Callus area ($mm^2$) | Embryo-genesis (%) | No. cotyledonary embryos | Callus area ($mm^2$) |
| Control | | 17±4[z] | 0.04±0.03 | 90 | 24±2 | 0.2±0.1 | 54 |
| CW | 1% | 16±4 | 0.2±0.2 | 70 | 11±3 | 2.2±2.2 | 49 |
| (v/v) | 5% | 15±2 | 2.0±0.8 | 94 | 19±4 | 0.5±0.3 | 79 |
| | 10% | 13±4 | 1.6±0.6 | 80 | 55±2 | 3.3±0.8 | 88 |
| | 20% | 49±5 | 3.0±1.1 | 89 | 74±5 | 13.7±1.7 | 134 |
| Proline | 0.1 | 2±2 | 0 | 65 | 41±6 | 0.5±0.3 | 19 |
| (mM) | 1.0 | 16±6 | 0.4±0.2 | 70 | 50±2 | 0.1±0.1 | 33 |
| | 10.0 | 51±10 | 0.2±0.2 | 33 | 26±3 | 0.1±0.1 | 16 |
| | 100.0 | 35±9 | 0.5±0.5 | 28 | 10±4 | 0 | 17 |
| adenine | 0.1 | 24±4 | 0.8±0.3 | 43 | 31±7 | 0.5±0.4 | 29 |
| sulfate | 1.0 | 34±6 | 1.1±0.4 | 48 | 42±6 | 0.1±0.1 | 32 |
| mg/liter | 20.0 | 9±3 | 1.0±0.3 | 67 | 42±6 | 1.1±0.4 | 34 |
| | 80.0 | 0 | 0 | 62 | 39±8 | 0 | 22 |
| $(NH_4)_2SO_4$ | 6.25 | 3±2 | 0 | 51 | 62±3 | 0.1±0.1 | 42 |
| (mM) | 12.5 | 3±3 | 0.1±0.1 | 54 | 69±3 | 0 | 24 |
| | 25.0 | 3±3 | 0 | 65 | 65±3 | 0.3±0.2 | 36 |
| 2,4-D | 0.01 | 1±1 | 0 | 81 | 32±5 | 0.5±0.2 | 34 |
| mg/liter | 0.10 | 1±1 | 0 | 87 | 40±6 | 0 | 25 |
| | 1.0 | 2±2 | 0 | 83 | 20±8 | 0 | 9 |
| BA | 0.01 | 7±4 | 0 | 74 | 35±7 | 0.3±0.2 | 30 |
| mg/liter | 0.10 | 2±2 | 0 | 83 | 35±10 | 0.6±0.3 | 12 |
| | 1.0 | 7±5 | 0 | 18 | 0 | 0 | 5 |
| $GA_3$ | 0.01 | 4±3 | 0 | 60 | 22±5 | 0.4±0.2 | 16 |
| mg/liter | 0.10 | 13±2 | 0.1±0 | 92 | 14±5 | 0.2±0.1 | 17 |
| | 1.0 | 11±3 | 0 | 97 | 19±4 | 0.2±0.1 | 30 |

[z] SE computed from transformed data (arcain).

** Data obtained 8 weeks in semisolid media. Each treatment consisted of 10 observations with 50 observations in the control.

The response depended on clone after 6 weeks in culture. BC 36 had the maximum response in terms of embryogenesis and number of cotyledonary embryos at 20% (Table 2), while BC 46 responded most at 10%. BC 5 produced greenish non-embryogenic structures at 10 and 20% CW.

**Table 2.** Effect of CW on embryogenesis in callus cultures[z] of BC 5, 36 and 46.

| CW (%) | BC 5 | | BC 36 | | BC 46 | |
|---|---|---|---|---|---|---|
| | Embryo-genesis (%) | No. cotyledonary embryos | Embryo-genesis (%) | No. cotyledonary embryos | Embryo-genesis (%) | No. cotyledonary embryos |
| 0 | 0 | 0 | 0 | 0.1±0.1 | 1±1 | 0.2±0.1 |
| 10 | 0 | 0 | 10±2 | 2.1±0.4 | 4±1 | 1.1±0.4 |
| 20 | 0 | 0.1±0.1 | 13±3 | 3.3±1.1 | 1±1 | 0.3±0.2 |

[z]Each treatment consisted of 5 petri plates with 4 callus clumps per plate.

The effect of $GA_3$ at 1.0 mg/liter and 2,4-D at $10^{-3}$ mg/liter and their combination was tested in a separate experiment (Table 3) BC 5 and 46 by about 2 folds as compared to the control but less so in BC 36. Although 2,4-D alone slightly increased embryogenesis in BC 36 and 46, it was reduced in BC 5. However, the combination of $GA_3$ and 2,4-D increased embryogenesis in BC 5 and 46 but not 36. There was no evidence that $GA_3$, 2,4-D or their combination affected embryo development.

**Table 3.** Effect of $GA_3$ and 2,4-D on embryogenesis in callus cultures of clones BC 5, 36 and 46.

| $GA_3$ mg/liter | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0 | | | | | 1.0 | | |
| Clone | 2,4-D mg/liter | Embryo-genesis[z] (%) | No. cotyledonary embryos[y] | Area ($mm^2$) | Embryo-genesis (%) | No. cotyledonary embryos | Area ($mm^2$) |
| BC 5 | 0 | 11±1 | 0.05±0.02 | 66 | 21±2 | 0 | 77 |
| | $10^{-3}$ | 9±2 | 0.03±0.02 | 76 | 17±3 | 0 | 69 |
| BC 36 | 0 | 22±3 | 0 | 20 | 34±3 | 0.2±0.1 | 26 |
| | $10^{-3}$ | 25±3 | 0.1±0.1 | 21 | 26±3 | 0.2±0.1 | 23 |
| BC 46 | 0 | 24±4 | 0.9±0.4 | 57 | 44±4 | 0.6±0.4 | 60 |
| | $10^{-3}$ | 29±4 | 0.8±0.4 | 54 | 42±3 | 0.9±0.4 | 59 |

[z]Mean estimate of the ratio of globular structures to callus × 100.
[y]Mean number of cotyledonary embryos per clump ± SE.

In a preliminary experiment, it was noticed that CW increased embryogenesis in budding centers of BC 46 and appeared to rejuvenate cultures. This experiment was repeated in liquid and semisolid media using both rejuvenated and unrejuvenated cultures of BC 46. Budding centers (25 $mm^2$ each) were transfered to either liquid or semisolid media containing 0, 10, or 20% CW. There were 4 budding centers per each culture flask (25 ml/125 ml flask) or petri plates (10 ml/15×60 mm). Cultures in liquid media were harvested after 10 days while those in semisolid media after 30 days. Cotyledonary embryos 1-10 mm in length were counted and the requency of embryos greater than 2 mm was determined (Fig. 2).

Coconut water enhanced embryo development in both liquid and semisolid media (Table 4) but the effect was greater in liquid than in semi solid media. In liquid culture the effect was greater in cultures previously rejuvenated with CW but there was no difference in semisolid cultures. Rejuvenated cultures developed better than non-rejuvenated even in the absence of CW, with the greater effect in liquid than semisolid media.

**Table 4.** Effect of rejuvenation (CW) and media state on embryo development in BC 46.

| CW (%) | Nonrejuvenated | | | Rejuvenated | | |
|---|---|---|---|---|---|---|
| | No. embryos | Embryos > 2 mm (%) | Avg.[z] size (mm) | No. embryos | Embryos > 2 mm (%) | Avg.[z] size (mm) |
| | Liquid medium (10 days) | | | | | |
| 0 | 101 | 0 | 1.1 | 244 | 16.0 | 1.7 |
| 10 | 210 | 18.0 | 1.7 | 413 | 34.0 | 2.4 |
| 20 | 257 | 27.0 | 1.8 | 356 | 31.0 | 2.1 |
| | Semisolid medium (30 days) | | | | | |
| 0 | 69 | 0 | 0.7 | 269 | 3.0 | 1.2 |
| 10 | 309 | 13.0 | 1.5 | 682 | 10.0 | 1.5 |
| 20 | 485 | 10.0 | 1.5 | 883 | 11.2 | 1.5 |

[z] Average length (mm) of embryo counting all embryos 1-10 mm length.

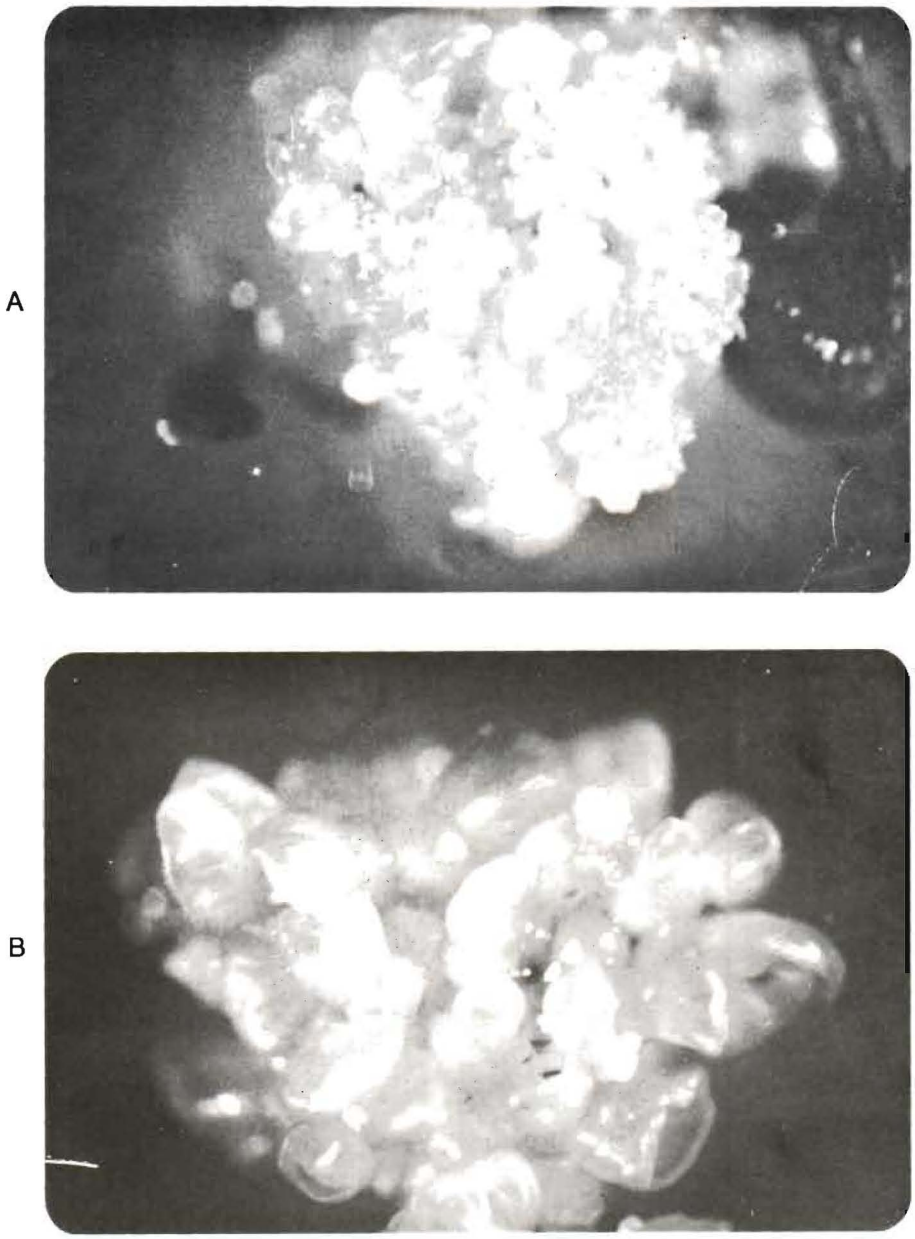


**Fig. 2.** A) Budding centers (BC 46) before treatment with coconut water.
B) Asexual embryos arising from BC 46 previously rejuvenated with CW.

## Discussion

Asexual embryogenesis from callus has been regarded as consisting of two separate stages: a callus initiation stage and an embryo induction stage. The callus initiation stage usually required an exogenous supply of auxin and/or cytokinin (Evans *et al.* 1981), whereas the embryogenic induction stage may or may not require auxin in the culture medium. A great many systems appear to involve the addition and subsequent withdrawal of auxin to stimulate embryogenesis. However, in some species, for example, with some members of the Poaceae (grass family), initiation and maturation of asexual embryos occur in the primary culture in the presence of auxin (Thomas *et al.* 1977, Vasil and Vasil 1981 a,b and Lu and Vasil 1982).

A range of growth factors has been shown to influence the induction stage. For example, embryogenesis has been enhanced with reduced nitrogen provided in the form of ammonium ion in carrot (Halperin 1966, Dougall and Verma 1978), alfalfa (Walker and Sato 1981), and egg plant (Gleddie *et al.* 1983) and when provided in the form of aminoacids as in carrot (Kamada and Harada 1979) and rice (siriwardana and Nabors 1983). Adenine sulfate enhanced embryo development in soybean (Phillips and Collins 1981). The effect of carbon source and concentration on embryogenesis has been investigated in *Theobrome cacao* (Elhag, *et al.* 1987) and results indicate that glucose and fructose stimulate embryogenesis from embryogenic competant callus as compared to sucrose.

The results of the present investigation indicated that a number of growth factors affected embryogenesis from callus with a distinct clonal response. Exogenous auxin (2,4-D) is not essential for the induction of embryogenesis but low 2,4-D concentration enhanced embryogenesis in clone BC 36. The response to reduced forms of nitrogen, ammonium and proline, in semisolid medium differed by clone and concentration. However, when these two factor were tested in liquid media (Elhag 1985), only one clone, BC 36, responded to the ammonium treatment. Proline treatment reduced embryogenesis in both clones in liquid media suggesting that the discrepancy in the response to proline in liquid media suggesting that the discrepancy in the response to proline may be due to medium state but conclusions are difficult to make because there appeared to be a clonal difference. The embryogenic response of BC 5 to GA confirms the report by Kononowicz and Janick (1984).

The present results confirm earlier observation by Pence *et al.* (1980) that CW and liquid medium enhanced embryo development in cacao. These results also support our earlier observation (Tables 1,2) that CW promoted embryogenesis from callus and budding centers. The stimulating component in CW has not been identified in our experiment but could be due, in part, to its high content of glucose

and fructose (Tulecke *et al.* 1961) which we have observed stimulates embryogenesis (Elhag *et al.* 1987).

The fact that so many factors affected embryogenesis suggests that the embryogenic response is indirect, perhaps *via* the control of cell division or aging as suggested by Linser and Neumann (1968).

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# أثر الهرمونات والعوامل الغذائية على إنتاج أجنة جسدية (Asexual embryos) من خلايا «وكدبات» الكاكاو *(Theobroma cacao)*

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تحتوي ثمرة الكاكاو على البذور وهي المستخدمة في الطب والتغذية لاحتوائها على القلويدات وعناصر غذائية أخرى. تطحن البذره بعد حمصها ويستخلص منها زبدة الكاكاو ومسحوق الكاكاو وهو المادة الأساسية في صناعة الشيكولاته.

لقد اثبتت الأبحاث ان الاجنة الجنسية *(Zygotic embryos)* حديثة التكوين يمكن ان تستكمل نموها خارج البذرة الأم وداخل انبوبة زجاجية معقمة بها مواد غذائية ويستخلص منها نفس المواد المستخلصة من البذرة الحقيقية (Pence *et al.* 1980, 1981). وثبت أيضاً ان الأجنة الجنسية يمكن التحكم في نموها وتحولها لإنتاج أجنة جسدية (Asexual embryos) بطريقة مستمرة على اوساط صناعية وهي أيضاً يمكن ان تستكمل نموها داخل انبوب *(in vitro)* (Kononowicz and Janick, 1984)

تناقش هذه الورقة انتاج اجنة جسدية من خلايا وكدبات (Callus) أجنة الكاكاو وتتناول أثر العوامل الغذائية والهرمونات على انتاج ونمو الأجنة الجسدية.

استعملت وسائط موراشيقي واسكوج كبيئة أساسية شبه صلبة بالإضافة لعدة عوامل غذائية وهرمونية أخرى لمعرفة أثرها على انتاج وتكوين خلايا جينية (embryogenic competent cells) من ثلاث سلالات من الخلايا. لقد تبين من هذا البحث ان هناك نوعان من الخلايا: خلايا جنينية وأخرى غير جنينية. الخلايا الجنينية ذات لون مصفر وملمس خشن وهي التي تكون الأجنة. أما النوع الآخر فهي ناعمة وذات لون مائي ولا تنتج أجنة.

وقد لوحظ عند التغيير الكمي والكيفي للعوامل المساعدة للنمو (غذائية وهرمونية) تبايناً في نسب الخلايا المتحولة لأجنة وان هذا التباين يرتبط ارتباطاً وثيقاً بأصل السلالات التي أخذت منها الخلايا. وثبت أيضاً ان بعض العوامل الهرمونية والغذائية يمنع تكوين الخلايا المتحولة لأجنة جسدية.