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HISTOLOGICAL CHANGES ASSOCIATED WITH SHOOT REGENERATION IN THE LEAF EXPLANTS OF *CLITORIA TERNATEA* (LINN.) CULTURED *IN VITRO*

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The leaf explants of *Clitoria ternatea* (Linn) when cultured on Shoot induction medium (SIM) i.e. Murashige and Skoog (MS) basal medium containing 2 mg l^{-1} Kinetin KN with 100 mg l^{-1} Adenine sulfate, periclinal divisions were initiated in the epidermal cells. In the periclinal divisions, some of the daughter cells formed the target cells which divided both anticlinally and periclinally to form cell division centers (meristemoids), precursors of adventitious shoots. The periclinal divisions in epidermis cells represents the dedifferentiation phase during which target (competent) cells are formed. Finally these periclinal divisions of the cells induces both dedifferentiation and shoot induction in the presence of exogenous plant hormones.

Keywords : Cell division, *Clitoria ternatea* (Linn.). Shoot regeneration.

Introduction

It has been shown that isolated explants are not immediately responsive to inducing signals but acquire that ability i.e., cellular competence during the culture period[1-3]. In plant tissues, competent cells are recognized as cells which respond to external signals to enter a specific developmental pathway[4,5]. In addition, many workers also suggested that cellular competence is acquired through the process of dedifferentiation[6-8]. In many cases cellular competence for regeneration is closely related to the occurrence and positions of cell divisions eg. for bud regeneration in stem segments and in the epidermis of *Torenia* [9,10]. But in other cases cellular competence for embryogenesis or organogenesis is not directly proportional to the level of mitotic activity of cultured tissues[11-13]. Therefore, the present study examines the ontogeny of shoot organogenesis in cultured leaf explants and the objective was to determine histological changes associated with shoot regeneration in the leaf explants of *Clitoria ternatea* (Linn.), a fast growing legume valued for its forage and medicinal importance. The plant is considered a good brain tonic and is useful in throat and eye infections, skin diseases, urinary troubles even in cattle, ulcer, antidotal and in improving memory. Roots are emetic used by the tribals to cause abortion an root paste yields an alkaloid called as *Clitorin* (MP 235°C).

Materials and Methods

Mature seeds of white flowered variety of *Clitoria ternatea* (Linn.) collected from the Karnatak University Botanical Garden were surface sterilized in 70% ethanol for 3 min, immersed in 0.1% $HgCl_2$ for 5 min, rinsed three times with sterile double distilled water, and germinated aseptically on Murashige and Skoog[14] (MS) basal medium containing 2.0% sucrose and 0.7% agar (Difco-bacto) at 25 ± 3° C for 16 hour photoperiod under cool white fluorescent light (100 µmol m^{-2} s^{-1}) with a relative humidity of 55 - 60%, From 15 - day - old aseptically grown seedlings, 1-2 cm long leaves were cultured on MS basal medium supplemented with 2 mg/l^{-1} Kinetin (KN) + 100 mg/l^{-1} Adenine sulfate (AS) (Shoot Induction Medium) (SIM). Leaf explants were cultured with both surface (adaxial or abaxial surface in contact with the culture medium) on shoot induction medium to study the orientation of leaf on shoot regeneration. Three replicates of 25 cultures each were raised in one set of experiment and experiment was repeated for 3 times. Thus, the total number of cultures raised in each experiment was 225. In case of control,

the leaf explants cultured on MS basal medium without growth regulators.

Histological Preparation of Leaf Explants

The leaf explants were fixed in FAA (Formaldehyde solution - 5ml, Glacial acetic acid - 5 ml, 70% Ethyl alcohol - 90ml.) After dehydrating through ethanol - butanol series and embedding in paraffin, they were sectioned (10 μM thick). The sections were stained with 1% safranin for 20 min, counter stained with 0.5% fast green for 15-20 min, and examined microscopically for the histological changes during the development of shoot buds.

Results and Discussion

On the basal MS medium the leaf explants showed shoot regeneration accompanied by callus formation. The percentage of responsive explants was only 20; the explants which did not produce shoot buds remained green for 15 days and finally necrosed (Fig. 1-A, B, C). Further development of the shoot buds did not occur on the basal medium. Explants in which the abaxial surface was in contact with the culture medium did not form shoot buds. Shoot regeneration and callus formation occurred in 60% cultures on SIM medium in 15-20 days after culture irrespective of which surface of the explant was in contact with the medium (Fig. 1 - D, E, F). The number of shoots per regenerating explant increased with longer exposure to MS supplemented medium; maximal shoot number was obtained in 20-day-old cultures (Fig. 1-G, H). This result concurs with previous results on certain other systems which showed that a period of cellular competence was required by cultured tissue[1-3]. Cellular competence for shoot regeneration is rapidly lost in cotyledons of *Pinus strobus* and *P. ponderosa* cultured on growth regulator-free medium[15-16]. In our study also, the dealy in treatment of the leaf explant with kinetin and adenine sulphate decreased the percentage survival of the explants; however, the explants that survived were able to regenerate shoots. This indicates cellular competence for shoot regeneration is not lost in the leaf explants of *Clitoria ternatea* in the absence of exogenous plant growth substance. Attfield and Evans[1] suggested that cellular competence in culture might depend on the mode of regeneration. Shoot regeneration in both *Convolvulus arvensis* and *Nicotiana tabacum* was obtained via callus formation and required prior cellular competence[1-2]. In our study also shoot regeneration occurred not directly but via callus indicating that the requirement for cellular competence is limited to callus formation. Furthermore, the ability for regeneration is not lost in cultured and surviving tissue on growth substance-free medium.

The leaf explants of *Clitoria ternatea* (L.) had a single layered epiermis. The leaf had a single layer of loosely arranged mesophyll cells which contained numerous chloroplasts and multilayered spongy mesophyll with larger intercellular spaces (Fig. 1-I, J). The culture of leaf explants on the MS basal medium did not induce periclinal division. Howerver, once the tissues were transferred to SIM, many epidermal cells divided periclinally within 5 days in adaxial and epidermal layers. Meristemoids were found in both epidermal layer after 5 days of hormonal exposure and with continued cell divisions. The cultured leaf explants required 10 days of hormonal exposure for shoot regeneration. Cell division centers (meristemoids) were formed from daughter cells of periclinal divisions. By day 20 of culture bud primordial (protrusions of actively dividing cells) were observed on the epidermal layers. It is also observed that only some of the derivatives of periclinal division resulted in the target cells for shoot inductions. The site of the target cell could be identified once the cell became a cell division center. The target cell usually the daughter cell at the surface of the dividing epidermal cell, then undergo both anticlinal and periclinal divisions to form a cell division center (Fig. 1-I, J).

On the basis of the histological observations it is found that adventitious shoots produced from the leaf explants of

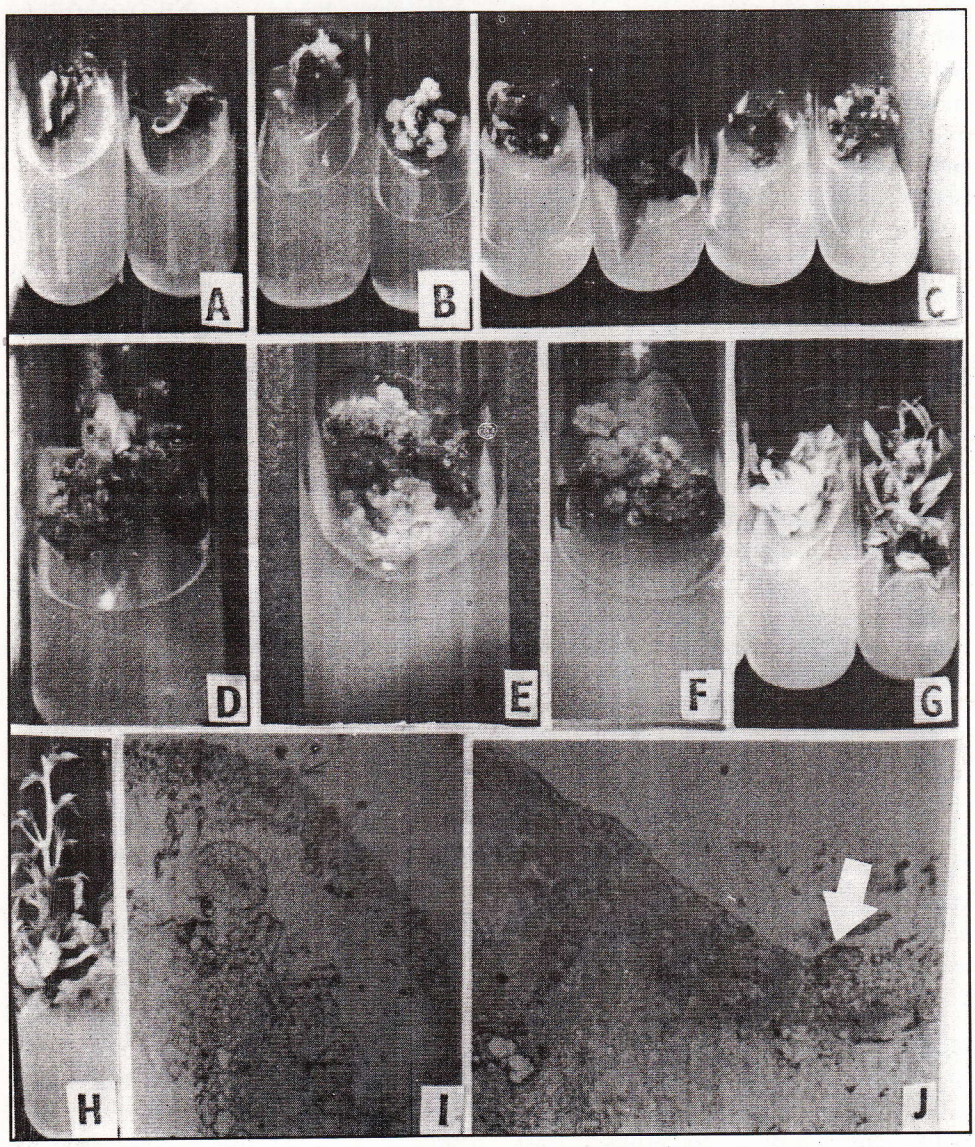

Fig. 1. Regeneration of shoots from leaf explants of *Clitoria ternatea* (L.)
A - Leaf explant cultured on MS basal medium (Control). **B, C** - Leaf explants showing shoot buds with callus on basal medium (BM) alone. **D, E, F** - Leaf explant showing shoot buds with callus on SIM. **G, H** - Luxurient growth of shoot buds on SIM. **I, J** - Histological preparation showing initiation of shoot buds. Epidermal origin of shoot buds.

Clitoria ternatea (L.) originate from epidermal cells. The epidermal cell origin of adventitious shoots has also been reported in earlier studies but the details of cellular origin were not available[17-20]. The present study also revealed that leaf epidermal cells do not directly regenerate into adventitious shoots. Initially perinclinal divisions are required and then a daughter cell (target cell) from one of the periclinal divisions divides both periclinally and anticlinally to form a cell division center (meristemoid), the

precursor to an adventitious shoot. The target cell is considered the competent cell for shoot induction and the formation of target cells may be interpreted in terms of cellular competence. Since epidermal cells in an intact plant generally divide anticlinally, periclinal divisions of epidermal cells in culture indicates a change in the cell polarity and development pattern of these cells. This change possibly represents the dedifferentiation phase. In addition, the present study also showed that not all periclinal divisions result in target cells. Although extensive periclinal divisions occurred in the adaxial epidermis, many of them did not lead to meristemoid formation. A special type of cell division such as unequal cell division or divisions at a preferential phase may be required for meristemoid formation[8,10]. In our present study, both unequal and equal periclinal divisions were observed in the epidrmal layers, but there is no basis to suggest that only one type of divisions result in target cell formation. The failure of meristemoid formation in epidermal cells with periclinal divisions may be due to *(1) Incomplete dedifferentiation (2) Competition among dedifferentiated cells in determining shoot origin, and/or (3) The inhibition of shoot induction by newly formed meristemoids in adjacent areas.*

The results also suggest that the cellular competence in the *Clitoria ternatea* leaf cultured consists of two distinct phases namely (1) *A reactivation phase (2) A dedifferentiation phase* comprises at least first 3 days of culture, during which no cytological changes are observed in cultured tissues even on SIM. Dedifferentiation, demonstrated by periclinal divisions, is then induced on SIM. Thus exogenous growth hormones are not required in the reactivation phase but are required for dedifferentiation. It is possible that there is an acquisition of hormone sensitivity during the reactivation phase. This sensitivity acquisition may be related to the availability of hormone-binding sites in the target tissues (i.e. Epidermal layers). Thus, once the sensitivity is acquired, the exogenous hormones act the signals for dedifferentiation and subsequent shoot induction.

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References

1. Attfield EM and Evans PK 1991, *J. Exp. Bot* **42** 59
2. Christianson M L and Warnick D A 1983, *Dev Biol*, **95** 288
3. Finstad, K Brown D C W and Joy K 1993, *Plant Cell Tissue & Organ Cult*, **34** 125
4. Meins F. Jr and Binns A N 1979, *Bio Science* **29** 221
5. McDaniel C N 1984, *Competence determination and induction in plant development.* In : Malacinski G M, Bryant S V (eds) Pattern formation, MacMillan New York, pp 393-412.
6. Halperin W 1973, *Can J Bot*, **51** 1801
7. Torrey J G 1977, *Hort. Science* **12** 14
8. Thorpe T A 1980, *Organogenesis in vitro : structural, physiological and biochemical aspects.* Int Rev Cytol (suppl IIA), : 71
9. Chlyah H, Tran Thanh Van M and Demarly Y 1975, *Plant Physiol* **56** 28.
10. Tran Thanh Van K 1981, *Annu Rev Plant physiol.* **32** 291
11. Joarder OI, Joarder NH and Date PJ 1986, *Theor. Appl. Genet.* **73** 286.
12. Barcelo P, Lazzeri P A, Martin A and Lorz H 1991, *Plant Sci.* **77** 243.
13. Dolezelova M Dolezel J and Nesticky M 1992, *Plant Cell Tissue & Organ Cult.* **31** 215.
14. Murashige T and Skoog F 1962, *Physiol. Plant.* **15** 473
15. Flinn B S, Webb D T and Newcomb W 1988, *Can. J. Bot.* **66** 1556
16. Ellis D D and Bilderback D E 1989, *Am. J. Bot.* **76** 348
17. Naylor E H and Johanson B 1937 , *Am. J., Bot.* **24** 673
18. Vazquez, A M and Short K C 1978, *J. Exp. Bot.* **29** 1265
19. Redway F A 1991, *Plant Sci.* **73** 243
20. Ohki S 1994, *Plant Cell Tissue & Organ Cult.* **36** 157