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Production and Developmental Pattern of Embryogenic Callus in *Oenanthe javanica* (Bl.) DC.

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미나리 體細胞 胚發生 캘러스의 獲得과 發達 形態

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This experiment was carried out to obtain embryogenic callus and to understand developmental mechanism of somatic embryogenesis in *Oenanthe javanica* (Bl.) DC. Experiments included the examination of explant source and media for embryogenic callus production and the observation of developmental pattern of embryogenic cells and non-embryogenic cells. Embryogenic calli were formed on zygotic pro-embryos together with their endosperms when they were cultured on MS media containing 1.0 mg/L 2,4-D. Embryogenic calli were also formed on the intact surface of in vitro grown stem or petiole segments after 6 - 8 weeks of culture, whereas non-embryogenic calli were formed on cut surfaces of the stem and petiole after 2 weeks of culture. Non-embryogenic calli were rhizogenic in suspension and solid media culture.

Key words: somatic embryogenesis, somatic embryos

Recognition of somatic embryogenesis in cultures, which can be used as a means of propagation, is one of the most significant findings in plant tissue culture. Since the embryogenesis from somatic tissues was observed by Steward et al. (1958) in carrot culture, investigations on somatic embryogenesis have been extensively and intensively carried out, and the number of species that have yielded somatic embryos in culture have greatly increased (Tisserat et al., 1979; Ammirato et al.). In order to obtain embryogenic callus which is used for mass production of somatic embryos, it is important to understand development mechanism of somatic embryogenesis related to explant source, plant growth regulators, and culture conditions. *Oenanthe javanica* (Bl.) DC. is regarded as one of the most potential vegetable crops for intensive cultivation, which specially requires uniform planting material. Therefore, we carried out this study to get the understanding on the mechanism of somatic embryogenesis and the information for mass propagation of *O. javanica* (Bl.) DC. through somatic embryos and report the results obtained from this experiment.

MATERIALS AND METHODS

Preparation of Explants

Oenanthe javanica (Bl.) DC. plants purchased from a local market were used as explant source. For in vitro material, fresh shoots were sterilized with a 2% sodium hypochlorite (Na_2ClO) solution for 10 minutes and the shoot tips from terminal and auxiliary buds were excised. The shoot tips were inoculated on MS (Murashige and Skoog, 1962) and 1/2 MS media to which BA (6-benzyladenine) was added at 5 concentration levels (0.1, 0.5, 1.0, 2.0, and 4.0 mg/L). In vitro shoots developed were maintained and increased by subculturing of the shoot tips and nodes every 3 weeks on MS basal medium solidified with 0.8% agar.

Induction and Proliferation of Embryogenic Callus

The cultured explants were leaf blade, stem segments, zygotic embryos, and zygotic pro-embryos together with endosperm for in vivo materials, and leaf blade, petiole, and stem segments for in vitro materials. The growth regulators of NAA, 2,4-D, IBA and BA each at 0.0, 0.1, 0.5, 1.0 and 2.0 mg/L were supplemented in various combinations to MS media. The media were solidified with 0.8% agar and adjusted at pH 5.8 prior to autoclaving. In vitro shoots which developed on MS basal medium were cut into 3 mm × 3 mm segments (leaf explants) and ca. 3 mm long segments (stem explants). For in vivo explants, leaves were cut into ca. 3 mm × 3 mm segments and young stems into ca. 4 mm long segments. The explants were cultured under continuous light (1,000 lux) or dark condition at 26 ± 1°C in culture room. After 6 weeks of culture, the cultures of each treatment were checked upon callus formation, and other characteristics such as fresh weight, friability, and color of the callus. The whitish-yellow embryogenic calli were separated from the non-embryogenic callus of variable types, and subcultured in liquid MS media supplemented with or without 2,4-D. The embryogenic calli were also maintained by subculturing on MS solid media containing 1 mg/L 2,4-D or by suspension culture in MS basal media.

Subculture of Non-embryogenic Callus

To examine the possible induction of embryogenic calli from non-embryogenic ones, the non-embryogenic calli induced from the cut surfaces of in vitro petiole segments cultured on media containing 2,4-D or NAA were transferred to solid media containing NAA, 2,4-D, IBA and / or BA each at 1 mg/L, and liquid basal media and subcultured every 4 weeks.

RESULTS AND DISCUSSION

Shoot Tip Culture for Explant Source

The shoot tips from terminal and axillary buds of *O. javanica* (BL.) DC. cultured for 3 weeks on MS or 1/2 MS media with BA at 5 concentration levels grew into 3 - 6 cm long shoots with 4 to 13 leaves (Table 1). As the BA concentration increased from 0.0 to 1 mg/L, the shoots became shorter and thicker. When the concentration of BA was 2 mg/L, the leaves became pale green. Number of shoots was similar but the shoots were longer and had more leaves on MS media than on 1/2 MS media. The in vitro shoot tips and nodes were repeatedly subcultured for further increase of the in vitro material. Therefore, it was shown that the media suitable to obtain a large amount of good shoots from shoot tip culture were original strength MS media supplemented with 0.1 to 0.5 mg/L BA.

Table 1. Effects of media and BA on shoot and root development from shoot tip culture of *O. javanica* (BL.) DC. on MS and 1/2 MS media for 4 weeks.

Medium	BA Concentration (mg/L)	Shoot		Leaf		Root	
		Number	Length(cm)	Number	Color[a]	Number	Length[b]
MS	0.0	2.5	5.0	4.2	G^{+++}	5.0	L^{+++}
	0.1	3.0	6.6	5.5	G^{+++}	8.0	L^{++}
	0.5	3.5	5.5	13.5	G^{++}	7.2	L^{++}
	1.0	3.0	5.0	8.2	G^{++}	6.0	L^{+}
	2.0	4.0	3.3	10.3	G^{+}	6.5	L^{+}
1/2 MS	0.0	2.0	3.0	5.0	G^{+++}	5.5	L^{+++}
	0.1	3.0	3.2	10.0	G^{++}	5.4	L^{++}
	0.5	5.0	3.7	9.5	G^{++}	7.0	L^{++}
	1.0	3.5	2.4	8.0	G^{++}	5.4	L^{++}
	2.0	4.4	1.6	11.0	G^{+}	4.8	L^{+}

[a] G^{+}: Light green, G^{++}: green, and G^{+++}: dark green.
[b] L^{+}: Short (< 1cm), L^{++}: medium (1 - 2 cm), and L^{+++}: long (> 2cm).

Induction of Embryogenic Callus

Calli were formed on in vivo stem and leaf explants when they were cultured on the media containing NAA or 2,4-D but not on media containing BA. The callus, however, was non-embryogenic and did not form any embryos (Table 2). The induction and growth of callus from the culture of in vitro leaf explants varied by different kinds and concentrations of growth regulators in the media as well as illumination conditions. Callus formation and growth was much more on the media containing NAA, IBA or 2,4-D singly than on media containing BA in combination with one of auxins (Table 3). The callus developed from leaf explants, regardless of culture media and conditions, was non-embryogenic. That is, in vitro cultured leaf explants was not seemingly suitable to obtain embryogenic callus.

Calli were formed on stem segments excised from in vitro cultured shoots on all the media containing growth regulators in various combinations. Most of the calli were non-embryogenic, however, the explants cultured on the media containing 0.5 mg/L NAA and 0.5 - 1.0 mg/L 2,4-D

Table 2. Effects of BA, NAA and 2,4-D on callusing and rhizogenesis in the culture of leaf and stem segments of *O. javanica* (BL.) DC. on MS medium under continuos light for 8 weeks.

Growth regulator		Number of explants inoculated	Callusing[a]		Rhizogenesis[b]		Senescence[c]	
	(mg/L)		Leaf	Stem	Leaf	Stem	Leaf	Stem
BA	0.5	12	-	-	-	-	-	-
	1.0	12	-	+	-	-	-	-
NAA	0.5	12	+	++	++	++	++	+
	1.0	12	+	++	+++	++	++	++
2,4-D	0.5	30	+	+++	+	++	++	+
	1.0	30	++	+++	+	++	+	+

[a, b] -: none, +: little, ++: a little and +++: much, [c] -: non, +: low, ++: medium, and +++: high.

Table 3. Effects of NAA, 2,4-D, IBA, and BA on the characteristics of callus induced from the culture of in vitro leaf explants of *O. javanica* (BL.) DC. under light and dark conditions for 6 weeks.

Auxin (mg/L)	BA (mg/L)	Callus: Formation (%)		Diameter (mm)		Color[a]	
		Illumination					
		Light	Dark	Light	Dark	Light	Dark
0.0	0.0	-	-	-	-	-	-
	0.1	-	-	-	-	-	-
0.1	0.0	-	-	-	-	-	-
	0.1	60	60	3.0	3.0	YG	YG
NAA 0.5	0.0	20	-	5.0	-	B	-
	0.1	30	60	8.0	3.0	G	Y
1.0	0.0	20	-	5.6	-	B	-
	0.1	30	80	6.0	6.0	YG	WY
2.0	0.0	40	-	4.7	-	B	-
	0.1	40	80	5.0	6.0	YG	Y
0.1	0.0	-	-	-	-	-	-
	0.1	20	20	3.0	5.0	W	B
0.5	0.0	20	60	3.0	7.0	Y	WB
	0.1	40	100	4.0	8.0	YG	WY
2,4-D 1.0	0.0	30	30	5.0	7.0	W	B
	0.1	40	20	3.0	6.0	W	W
2.0	0.0	40	20	3.0	4.0	B	B
	0.1	90	40	4.0	3.0	YG	B
0.1	0.0	20	-	4.0	-	B	-
	0.1	20	40	7.0	8.0	B	YW
0.5	0.0	40	-	4.0	-	B	-
	0.1	30	60	4.0	5.0	B	YW
IBA 1.0	0.0	20	-	4.0	-	B	-
	0.1	-	100	-	6.0	-	B
2.0	0.0	60	-	4.0	-	B	-
	0.1	60	100	4.0	8.0	B	W

[a] B: brown, YG: yellowish green, WY: whitish yellow, and W: white.

Table 4. Effects of NAA, 2,4-D, IBA and BA on rhizogenesis and embryogenesis in the culture of in vitro stem segments of *O. javanica* (BL.) DC. on MS medium under continuos light for 6 weeks.

BA (mg/L)	Auxin (mg/L)[a]				
	0.0	0.1	0.5	1.0	2.0
	NAA				
0.0	-	R	R/E	R	R
0.1	-	R	R	R	R
0.5	-	C	R	C	C
	2,4-D				
0.0	-	C	R/E	R/E	C
0.1	-	C	C	C	C
0.5	-	C	C	C	C
	IBA				
0.0	-	R	R	R	R
0.1	-	R	R	R	R
0.5	-	C	C	C	R

[a] -: No response, R: root development, E: embryo formation and C: callusing only.

Table 5. Effects of NAA and 2,4-D on rhizogenic and embryogenic potential of the callus developed from petiole segment culture of *O. javanica* (BL.) DC. on MS media under dark condition for 6 weeks.

BA (mg/L)	Auxins (mg/L)[a]									
	NAA					2,4-D				
	0.0	0.1	0.5	1.0	2.0	0.0	0.1	0.5	1.0	2.0
0.0	N	N	R	R	R	N	E	E	E	E
0.1	N	N	E	N	N	N	E	E	E	E
0.5	N	E	N	N	N	N	E	N	N	N
1.0	N	N	N	N	E	N	E	N	N	N
2.0	N	N	N	N	N	N	N	N	N	N

[a] Data were collected in 6 weeks for rhizogenic potential and in 2 to 3 months for embryogenic potential. N: non-rhizogenic and non-embryogenic, R: rhizogenic but non-embryogenic, E: embryogenic. Embryogenic potential was determined by the formation of embryogenic clumps or embryos.

produced embryogenic calli on their intact surface (Table 4) (Figure 1B). The embryogenic callus was easily distinguished by its bright white-yellow color from non-embryogenic ones. The embryogenic calli formed from stem segments on the media containing NAA (0.5 mg/L) developed into embryos and then into plantlets. Non-embryogenic callus derived from the culture of stem segments on the media containing NAA, IBA or 2,4-D was more rhizogenic than that developed on media containing 2,4-D. The former was greenish, but the latter was yellowish or white.

From the in vitro petiole segment culture, callus

Figure 1. Formation of embryos and embryogenic callus from *in vitro* petiole segments of *O. javanica* (BL.) DC. on the media containing BA and 2,4-D. A: Development of somatic embryos (e) on the media containing 0.5 mg/L 2,4-D and 0.5 mg/L BA (60x). B: Formation of embryogenic callus (ec) on the media containing 1 mg/L 2,4-D, which was bright yellow and whitish-yellow in color. C: Development of somatic embryos on the intact surface in prolonged culture on the media containing 2,4-D and BA together (2 months). Development of embryos (e) and embryogenic callus from the intact surface (ep) was clearly shown when the non-embryogenic callus (nc) which was developed from cut surface of stem was removed.

development occurred regardless of growth regulators in the media (Table 5). Some of the petiole segments cultured on media containing 2,4-D, NAA and BA each at various levels produced two different kinds of callus (Figure 1A, B). The one developed first from cut surface of the explants in 2 weeks and the other developed from intact surface of the explants in 6 to 8 weeks. The former was non-embryogenic while the latter was embryogenic. Non-embryogenic callus which proliferated rapidly at the early stage covered whole surface of the explant, but embryogenic callus which originated from the intact surface of explants were protruded out of the non-embryogenic callus (Figure 1C). Embryogenic calli were derived from the intact surface of the petiole covered by non-embryogenic calli. It seems that the pre-embryogenic determined cells which already existed in explants produced embryogenic callus.

When 2,4-D were added to the solid medium, embryogenic callus of *O. javanica* (BL.) DC. proliferated without forming embryos. Embryogenic callus or embryogenic cells in intact surface of petiole segments did not readily precede embryogenesis on media supplemented with 2,4-D only, but continued to form embryogenic callus (Figure 1B). The embryogenic callus developed into embryos on the media containing 2,4-D and BA. That is, BA seemed to promote embryo development by eliminating the repressive action of 2,4-D on embryogenesis in the culture of *O. javanica* (BL.) DC (Figure 1C). Embryogenic callus, when left for a long time on the media containing 2,4-D, sometimes formed embryos.

As a standard sequence for induction of embryogenic callus, explants are generally inoculated on the primary medium containing 2,4-D (Ernest and Oesterhelt, 1984 ; Finer, 1988a, b ; Gmitter and Moore, 1986 ; Krogstrup and Erikson, 1988). The growth regulator, 2,4-D is effective on the induction and proliferation of embryogenic callus but repress the development of embryos from the embryogenic callus induced on solid media (Helperin and Witherell, 1964 ; Sung et al., 1979). Cytokinins were reported to be effective on the growth and development of embryos on solid media (Fujimura and Komamine, 1980).

Non-embryogenic callus induced from petiole segments on the media containing NAA was more rhizogenic, friable, and transparent than the non-embryogenic callus induced on the media containing 2,4-D. When more than 2 mg/L BA was added to the media containing 0.1 mg/L - 2 mg/L 2,4-D, callus proliferation was repressed and the callus degenerated, but parts of the callus maintained greenish. Differentiation of roots from the callus seemed to be indication of non-embryogenic ones. That is, the callus competent to produce roots was always non-embryogenic callus. When BA was added with NAA to the media, the callus induced from petiole segments became greenish when subcultured under light.

From the culture of zygotic pro-embryos with endosperm in ca. 20 days after anthesis on the media containing 1 mg/L 2,4-D, embryogenic callus developed after 2 months of inoculation. Once embryogenic callus appeared out of endosperm, it proliferated very rapidly, and produced embryogenic callus only (Figure 2A, B). Only endosperm culture without embryos did not form any callus, and became dark brownish and then degenerated to death. Therefore, the embryogenic callus developed from the culture of zygotic pro-

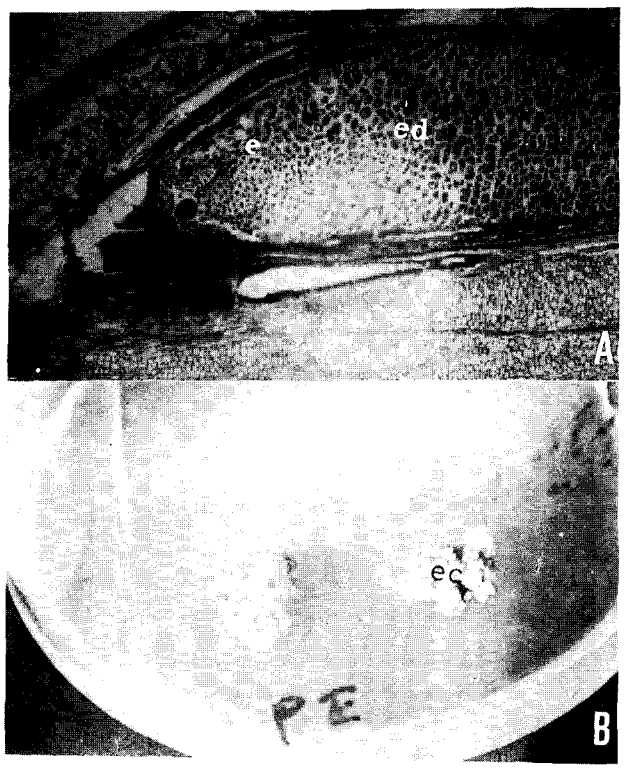

Figure 2. A zygotic pro-embryos of *O. javanica* (BL.) DC. and embryogenic callus developed from the pro-embryos. A: A longitudinal section of an ovary in 20 days after anthesis, showing pro-embryo (e) stained deep purple by hematoxylin and endosperm (ed) red by safranin, x200. B: The embryogenic callus (ec) developing from the zygotic pro-embryos cultured on MS medium containing 2,4-D at 1 mg/L.

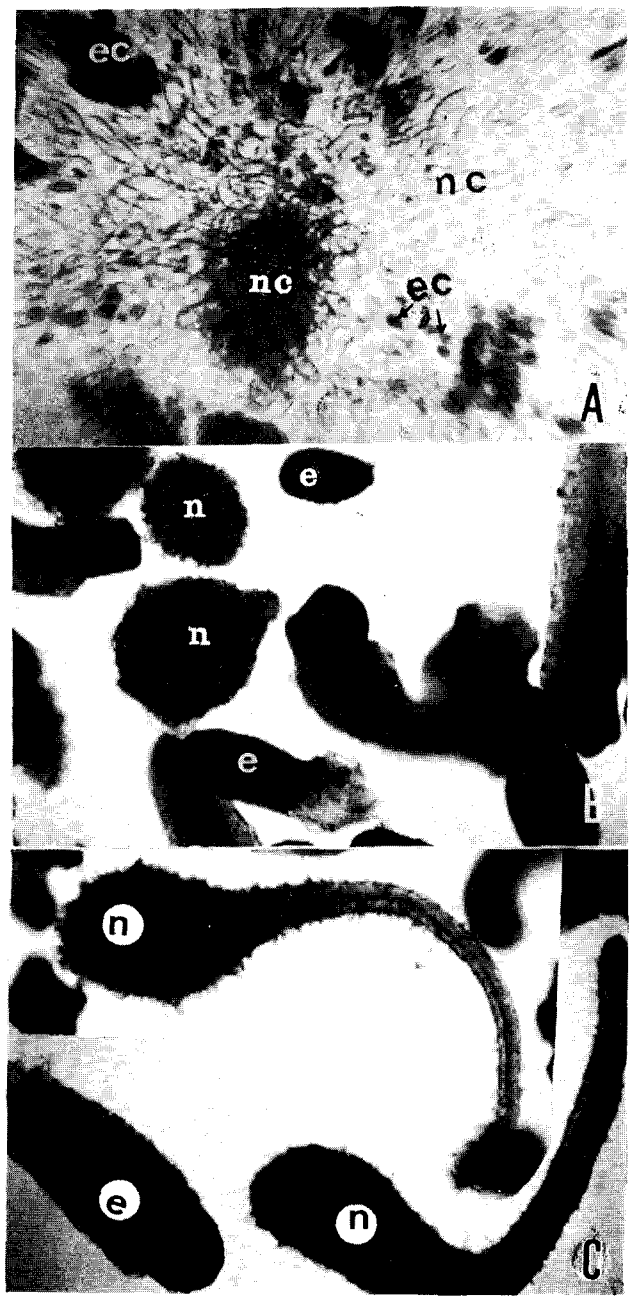

Figure 3. Different kinds of products from globular embryos of *O. javanica* (BL.) DC. in suspension culture with MS basal medium. A: Embryogenic singles and clumps (ec) and non-embryogenic cells and aggregates (nc) (200x). B: An embryo (e) and non-embryogenic cell aggregates (n) producing root (100x). C: An embryo of which plumule and radicle is clearly shown, and two non-embryogenic aggregates (n) with long roots developed from non-embryogenic cells (100x).

embryos with endosperm seemed to originate from the cells of pro-embryos.

Tisserat et al. (1979) and Sharp et al. (1980) proposed pre-determination (PEDC) theory in which the explant and certain physiological quality associated with it are most significant in determining whether embryo initiation can occur. They say that cells which undergo embryo initiation are embryogenic from the beginning, and that their culture in vitro simply provides the opportunity for embryogenesis to occur. It is considered that *O. javanica* (BL.) DC. showed the PEDC pattern in the formation of embryogenic callus and embryos. Embryogenic callus was induced very effectively by culturing zygotic pro-embryos and in vitro petiole segments.

Subculture of Embryogenic Callus and Non-embryogenic Callus

When embryogenic calli were sub-cultured in liquid media, they divided into small embryogenic cell aggregates, and then these aggregates developed into embryogenic clumps again. Some cells in the embryogenic clumps developed into embryos and the embryos were released into the suspensions.

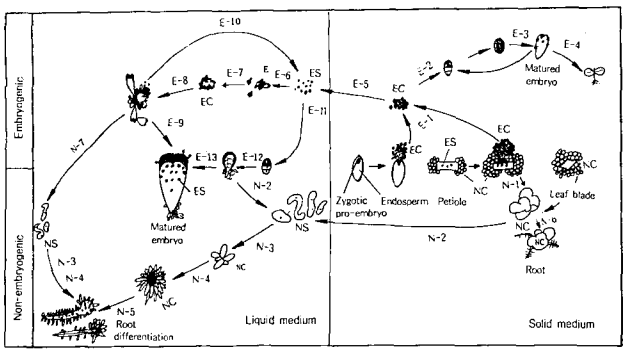

Figure 4. Postulated diagram representation on the development of embryogenic callus, somatic embryos and non-embryogenic cells in the culture of *O. javanica* (BL.) DC. E-1: Induction of embryogenic callus from zygotic pre-embryos or from intact surface of petiole or stem segments. E-2 / E-4: Embryo development from embryogenic callus on solid media. E-5: Proliferation of embryogenic cells. E-6 / E-7: Formation of embryogenic clumps, E-8 / E-9: Embryo development in embryogenic clumps, E-10: Release of embryogenic cells from embryogenic clumps, E-11/E-13: Embryo development from embryogenic cells. N-1: Induction of non-embryogenic callus from leaf blade, or cut surface of stem and petioles. N-2: Proliferation of non-embryogenic cells. N-3 / N-4: formation of non-embryogenic cell aggregates, N-5: Root development from non-embryogenic callus, N-6: Root differentiation from non-embryogeic callus. N-7: Release of non-embryogenic cells from embryogenic clumps, * NS: Non-embryogenic single cell, NC: non-embryogenic callus or clump, ES: embryogenic cells and EC: embryognic callus and clump.

Meanwhile, other cells in embryogenic clumps became non-embryogenic cells by advancement of clumps and these non-embryogenic cells became a main sources of non-embryogenic cells in the suspensions. The non-embryogenic parts such as radicles or root fragments of the developing embryos also seemed to produce non-embryogenic cells or cell aggregates (Figure 3A, B)

The non-embryogenic calli developed from the cut surface of petiole segments cultured on the media containing NAA or 2,4-D were left on the primary culture media for a prolonged period or subcultured on the media containing various growth regulators in oder to investigate possible induction of embryogenic callus from them. All of the non-embryogenic calli subcultured on solid media containing 2,4-D, NAA, IBA or BA at 1 mg/L each remained as non-embryogenic ones and continuously produced non-embryogenic callus and roots. Calli on the media containing BA were greenish, compact and growing rapidly, but they were also non-embryogenic and produced roots. Embryo development also did not occur from the non-embryogenic callus subcultured in liquid medium. Non-embryogenic cell aggregates derived from these calli were rhizogenic, and consisted of larger cells only (Figure 3A, B, C).

It was reported that in the Gramineae, non-embryogenic callus can be changed into embryogenic callus in culture. That is, non-embryogenic cell which is long and large converted into embryogenic callus by change of division patterns (Chen et al., 1985). Jones (1974) reported that isolated vacuolated carrot cells which was thought to be non-embryogenic callus can dedifferentiated and give rise to embryogenic clusters from which somatic embryos arises. Thus he suggested that non-embryogenic cells could converted into embryogenic ones. Halperin (1966), however, reported that non-embryogenic cells do not convert into embryogenic callus and the transition from vacuolated cell into embryogenic unit is rather a rare event. Smith and Street (1966) examined possible causes for loss of embryogenic potential in carrot cultures, and found that the larger cell were generally tetraploid and incapable of embryogenesis. Non-embryogenic cells of *O. javanica* (BL.) DC. were considered not to be due to genetic change because they induced in the primary culture or on the media free of growth regulators. In *O. javanica* (BL.) DC., non-embryogenic cells never produced embryogenic cells or embryos even though they were repeatedly subcultured in various media and conditions. Therefore, the embryogenic cells are unlikely induced from non-embryogenic callus at least in *O. javanica* (BL.) DC.

The overall results suggests that the development of embryogenic cells in the culture of *O. javanica* (BL.) DC. as illustrated in Figure 4. That is, embryogenic callus was induced only from the pre-existing embryogenic cells in the zygotic pro-embryos or intact surface of internodes and petioles of the in vitro grown shoots. The embryogenic callus cultured on solid media produced somatic embryos and plantlets. Meanwhile, embryogenic callus in suspension proliferated into embryogenic cells and clumps, and non-embryogenic cells and cell aggregates. The embryogenic clumps consisted of embryogenic cells, non-embryogenic cells and developing embryos. And non-embryogenic cells and aggregates were derived from embryogenic clumps in suspension culture. Non-embryogenic callus on solid media differentiate several roots and non-embryogenic cells in suspension culture became a cell aggregate which surface is covered with elongated cells, and then it differentiated a root (Figure 3A, B, C). Once the cells became non-embryogenic, they never returned embryogenic regardless of the cultural conditions.

摘 要

미나리의 體細胞 胚發生 條件 및 機作을 究明하고자 胚發生 캘러스의 獲得에 適合한 試料와 培地를 究明하고 胚發達의 過程을 觀察하여 본 結果, 器內 植物體 確保를 위한 미나리의 莖頂培養은 發生한 줄기 수와 生育狀態로 보아 BA가 0.1 - 0.5 mg/L 添加된 MS培地가 適合하였으며 胚發生 캘러스의 發生은 胚乳와 함께 採取·培養한 幼胚를 2,4-D가 1 mg/L 添加된 培地에서 2 個月間 培養하였을 때 發生하였다. 器內에서 生長한 줄기와 葉柄을 培養하였을 경우는 置上後 2週에 葉柄의 切斷 部位에서 發生한 캘러스는 모두 胚發生 캘러스가 아니었으나 置上 後 6 - 8주에 줄기와 葉柄의 表皮부위에서 發生한 캘러스는 胚發生 캘러스이었다. 胚發生 캘러스는 繼代培養에 의하여 大量 增殖이 可能하며 이들은 胚發生 캘러스와 非胚發生 캘러스를 同時에 形成하였다. 非胚發生 캘러스는 多樣한 生長調節劑를 添加한 液體 및 固體培地에 反復 및 遲滯培養하여도 胚發生 캘러스로의 分化는 일어나지 않았다. 非胚發生 캘러스는 固體培地에 培養하면 쉽게 增殖되어 뿌리를 分化하며, 液體培地에 懸濁培養할 경우에는 單細胞로 單離되고, 다시 이들은 작은 圓形細胞塊를 形成한 후 단지 뿌리만을 發生하였다.

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