Effect of 2, 4-Dichlorophenoxyacetic Acid and 6-Benzylaminopurine on Formation of Adventitious Buds and Somatic Embryos of Maize (*Zea mays* L.) in Relation to Internal Levels of Free and Conjugated Auxins.

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(Received May 3, 2005)

Abstract: *In previous studies, it was observed that shoot apex explants of maize cultured in vitro could be induced to form adventitious buds and/ or somatic embryos by modifying plant growth regulators content, auxin and cytokinin, in the culture medium. The objective of this investigation was to study the effects of 2,4-dichlorophenoxyacetic acid (2,4-D) in the presence of two different levels of 6-benzylaminopurine (BAP) on the in vitro differentiation of shoot apex explants of maize (*Zea mays *L.) and on the level of free and conjugated the natural auxin, indole-3-acetic acid (IAA), and the synthetic one, 2,4-D, during the time-course studied of morphogenesis. Shoot apex explants excised from 7-day-old seedlings of maize germinated in vitro were cultured on MS medium supplemented with 500 mg/L casein hydrolysate (CH), 5.0 and 20 µM BAP and 2.5 µM 2,4-D. Free and conjugated IAA and 2,4-D concentrations were analyzed by high performance liquid chromatography (HPLC). Dynamics of endogenous IAA and 2,4-D changes showed that lower level of the BAP promoted auxin induced somatic embryogenesis and its higher concentration stimulated another type of regeneration, bud organogenesis. Thus, the present results suggest that formation of adventitious buds is associated with a low concentration of free and conjugated auxins, IAA and 2,4-D and the reverse is true in relation to somatic embryo formation. Data also confirmed the importance of the exogenous application of auxin: cytokinin ratio in the medium in regulating maize plant growth and morphogenesis. The physiological roles of the auxin-like-regulator 2,4-D in inducing adventitious buds and somatic embryos formation are discussed.*

***Abbreviation**: IAA= indole-3-acetic acid; BAP= 6-benzylaminopurine; 2,4-D= 2,4-dichlorophenoxyacetic acid; MS= Murashige and Skoog; PGRs= plant growth regulators*

Key words: Free and conjugated auxins, maize, plant growth regulators, regeneration

INTRODUCTION

One of the characteristics of plant growth and development is that somatic cell differentiation is reversible. This can be best demonstrated in *in vitro* systems where somatic plant cells can regain their totipotency. Formation of adventitious buds, i.e. bud organogenesis, and somatic embryos, i.e. somatic embryogenesis, are evidence of this totipotency. Differentiated somatic cells are first de-differentiate, then acquire embryogenic potential and start a new life cycle via a series of characteristic morphological stages of development (Mordhorst *et al.* 1997). The acquisition of regeneration capacity is related to several intra- and extracellular factors; and different stimuli can trigger regeneration programs (De Jong *et al.* 1993). Plant regeneration protocol, e.g. somatic embryogenesis, has become an important model system for studying the de-differentiation and re-differentiation of plant cells, and is extensively used as an experimental system, instead of a zygotic system, for elucidating physiological, biochemical, and molecular biological events during embryogenesis (Zimmerman 1993, Schmidt *et al.* 1997). Plant regeneration protocol has also been applied in plant breeding and clonal propagation of various plant species.

Cultural systems of maize shoot apex explants could be induced to form adventitious buds and/ or somatic embryos by modifying cytokinin level in the presence of a synthetic auxin in the culture medium, as reported by Zhong *et al.* (1992), Zhang *et al.* (1998), and Sairam *et al.* (2003). It is not fully understood why somatic embryogenesis is induced in some cases while in others only adventitious buds formation is triggered (Thorpe 1994, Sharma and Thorpe 1995, Zhang *et al.* 1998, Ribnicky *et al.* 2002). Any possible correlation between internal free and conjugated IAA and 2,4-D levels and developmental origin of bud organogenesis and somatic embryogenesis is as yet undefined (Cooke *et al.* 1993, Thorpe 1994, Gai 2004).

In this investigation, shoot apex explants of maize (*Zea mays* L. cv. Honey N Pearl) seedlings grown *in vitro* were used to study the effects of exogenously applied BAP at two different levels in the presence of 2,4-D on inducing two different types of morphogenesis of shoot apex explants

during the time-course of bud organogenesis and somatic embryogenesis was also evaluated. The overall aim of this work was to study one of the major physiological and biochemical changes that may significantly affect maize plant morphogenesis

MATERIALS AND METHODS

Plant material and culture conditions

Mature seeds of sweet-corn (*Zea mays* L. cv. Honey N Pearl) provided by Illinois Foundation Seeds (Champaign, ILL., USA) were surface sterilized and germinated *in vitro* as described before (Tartoura 2005). Six days after germination, 5-mm-long segment of the seedling containing a shoot apex and stem proximal to the shoot apex was aseptically excised and cultured on MS (Murashige and Skoog 1962) medium with 3% sucrose containing 500 mg/L casein hydrolysate (CH), 20 and 5 µM BAP and 2.5 µM 2,4-D. These treatments efficiently induced adventitious buds and somatic embryos formation, respectively, based on preliminary experiments. Three shoot apices were cultured in a Petri dish, each containing 20 ml medium and the cultures were incubated at 24 ± 2 °C in darkness. Subcultures were made at 4 weeks intervals. The establishment of a stable adventitious buds and somatic embryo formation began with the retardation of leaf growth. Continuous removal of elongated leaves is critical for culture success. The effects of exogenously applied BAP and 2,4-D on morphogenetic pathways of shoot apex explants and on internal concentrations of IAA and 2,4-D were studied at subculture 2. Since the efficiency of formation of adventitious buds and somatic embryos became higher after two cultures, I chose this subculture for determination of morphogenesis and quantitation of auxins. Data are the mean values of three independent experiments, each consisting of 30 shoot apices in three replicates.

Quantitation of free and conjugated auxins

Determinations of auxins IAA and 2,4-D, in both free and conjugated forms, were performed in one sample according to a modified method of Ribnicky *et al.* (1996). Proliferating shoot apex material (5.0 g FW) was frozen in liquid nitrogen, powdered, and extracted with 90% methanol (MeOH, 10 ml g^{-1} FW) for 4 h at 4 °C in darkness. The tissues were re-extracted twice for 4 h each. The combined extracts were reduced to the aqueous phase *in vacuo* at 30 °C using a rotary evaporator and centrifuged at 24,000 g for 20 min. Supernatants were evaporated to about 0.5 ml, then 0.5 ml of MeOH was added and the extracts were passed through an activated C_{18} Sep-Pak cartridge (Purves and Hollenberg 1982), followed by elution with 5 ml of 90% MeOH: H_2O (v/v). The filtrate was divided into two parts, one for free IAA and 2,4-D and the other for total IAA (free + ester and amide conjugates) determination. Quantitations of IAA and 2,4-D by HPLC were achieved using a standard curve constructed by injection of authentic IAA and 2,4-D (Sigma) quantities and corrected for IAA and 2,4-D losses during extraction and purification by adding, in a separate experiment, defined amounts of each authentic auxin to homogenates and then quantify them by HPLC. The method used for extraction and purification was highly reproducible. The recovery rates were about 81% and 76% in terms of free and conjugated forms, respectively. IAA and 2,4-D conjugates were calculated by subtracting the free form values from their total amounts estimated after base hydrolysis. Identification of free IAA and 2,4-D released from their conjugations was confirmed by adding authentic IAA and 2,4-D to a part of the sample.

All chemicals used in this investigation including the basal medium were obtained from Sigma Co., St. Lous, Mo., USA.

RESULTS

Observation on morphogenetic pathways

Figure 1 shows that higher level of BAP (20 µM) in the presence of 2,4-D effectively induced adventitious bud formation on shoot apex explants cultured *in vitro* (Fig. 1A), whereas lower BAP concentration (5 µM) led to initiating another type of regeneration, i.e. somatic embryos, as shown in Fig. 1B. Thus, exogenous application of BAP concentrations in the presence of the synthetic auxin 2,4-D could regulate plant morphogenesis of *Z. mays*.

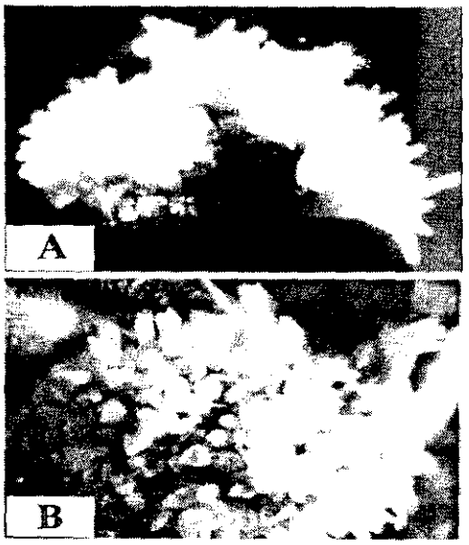

Fig. 1. Two types of morphogenetic pathways in shoot apex of *Z. mays*. Section from a meristematic dome cultured on MS medium, containing 500 mg/l CH, 20 µM BAP, and 2.5 µM 2,4-D, showing adventitious bud formation (A), somatic

embryogenesis from a shoot apex of pre-cultured on MS medium containing 500 mg/ l CH, 5 µM BAP, and 2.5 µM 2,4-D. X 16.

Internal free and conjugated auxins

Figure 2 shows that there was transient increase in endogenous free IAA in treated plant tissues during 48 h in response to 20 and 5 µM BAP plus 2.5 µM 2,4-D application, with the maximum increase occurred at the lower BAP concentration. Thereafter,

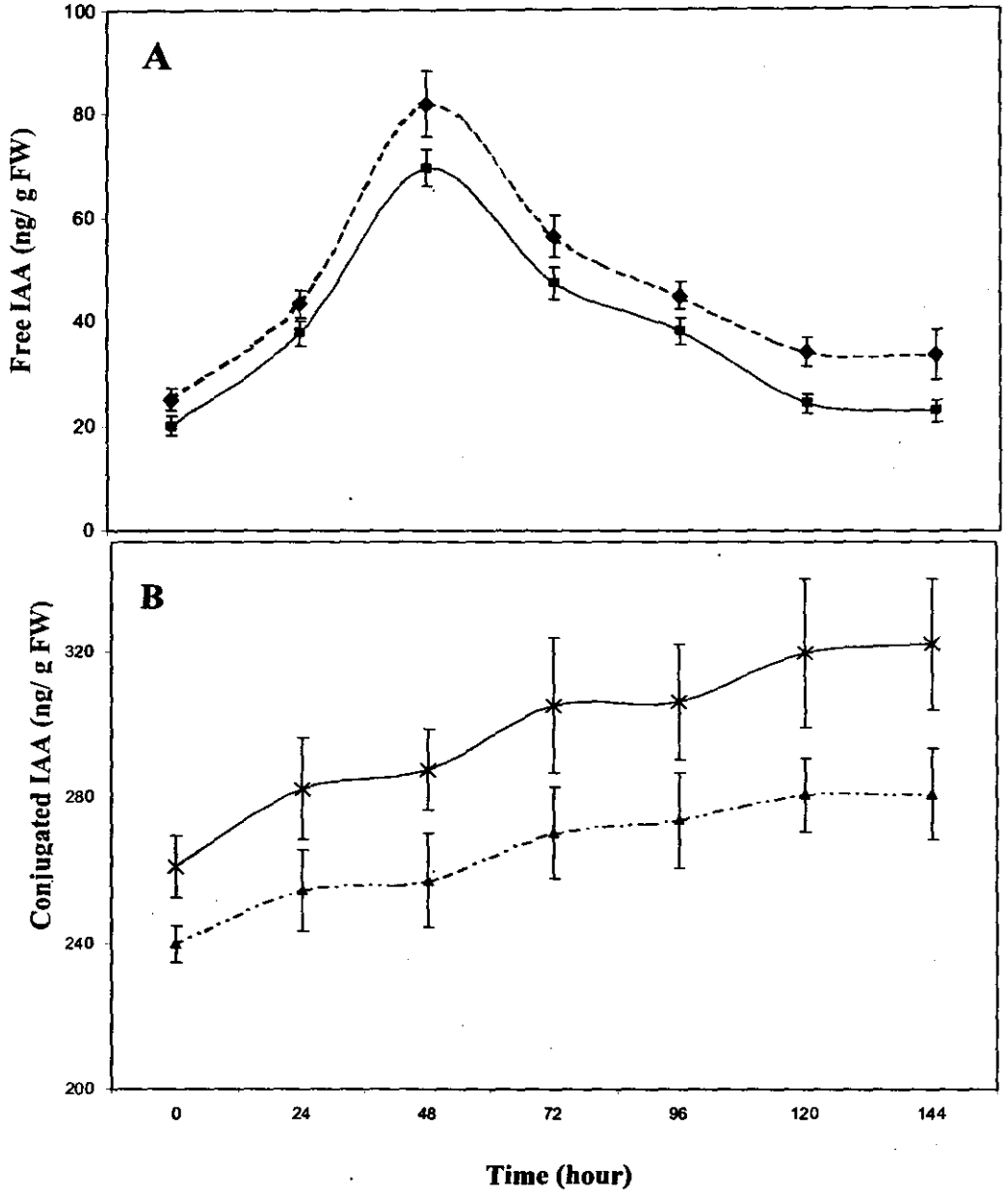

Fig. 2. Changes in endogenous free IAA (A) and conjugated IAA (B) in treated shoot apex explants of *Z. Mays* during the early events of the indicated time-course of regeneration process. Vertical bars represent ± SD.

Free IAA (—■—; 20 µM BAP plus 2.5 µM 2,4-D; ···◆···; 5 µM BAP plus 2.5 µM 2,4-D)

Conjugated IAA (-▲-; 20 µM BAP plus 2.5 µM 2,4-D; —✕—; 5 µM BAP plus 2.5 µM 2,4-D)

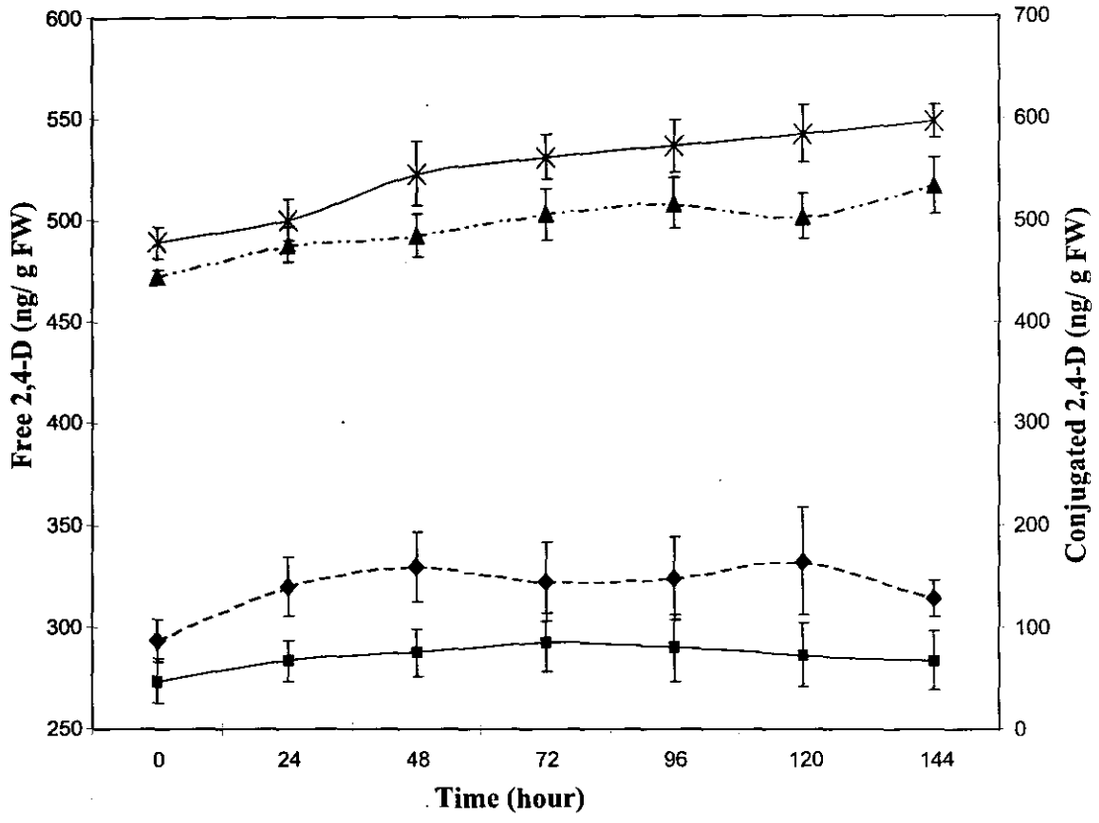

Fig. 3. Changes in endogenous free 2,4-D and conjugated 2,4-D in treated shoot apex explants of *Z. Mays* during the early events of the indicated time-course of regeneration process. Vertical bars represent ± SD. The symbols designations are the same as those in Figure 2.

a gradual marked decrease in IAA level was noted in 20 µM BAP treated shoot apices compared to those treated with the lower one (Fig. 2A). Figure 2 also shows that conjugated IAA levels were also increased in both treatments with a pronounced higher level in the tissues treated with lower than higher BAP concentration, indicating that exogenous higher BAP led to a decrease in conjugated IAA level and the reverse was true with lower BAP concentration as shown in figure 2B. In terms of free and conjugated 2,4-D, Figure 3 shows that internal free 2,4-D remained unchanged during most of the time-course studied in both treatments, with a higher level at 5 µM BAP. However, conjugated 2,4-D gradually increased during embryogenesis, as shown in figure 3. Based on the forgoing results, it was evident that higher BAP concentration caused a decrease in the total amount of auxins, IAA and 2,4-D, compared to the lower BAP concentration, indicating that formation of adventitious bud and somatic embryos (Fig. 1 A and B) are associated with lower and higher level of total auxins (Fig. 2 and 3), respectively.

DISCUSSION

Occurrence of *in vitro* plant morphogenesis is considered to be a result of an intricate balance of PGRs incorporated in the culture medium and those present within the tissues. In this study, *Z. mays* shoot apex explants have shown two morphogenetic pathways via producing adventitious buds and somatic embryos formation (Fig. 1). Data presented here indicate that, in the presence of 2.5 µM 2,4-D, 20 µM BAP application induced adventitious bud whereas 5 µM BAP induced somatic embryo formation (Fig. 1, A and B). These findings fully support previous studies conducted by Zhong *et al.* (1992), Zhang *et al.* (1998), and Sairam *et al.* (2003) who reported that maize plantlets regenerated from shoot meristem explants via formation of adventitious buds and somatic embryos. In addition, they illustrated a description of scanning electron microscopic analysis of *de novo* differentiation regenerated *in vitro* from shoot apices of maize. Further, they also reported that the number of regenerants was dependent on the size of enlarged meristematic domes with larger ones giving rise to greater numbers of regenerants. Their findings together with the data presented here clearly confirm the importance of the exogenous auxin: cytokinin

ratio in the culture medium in regulation *in vitro* maize regeneration. In terms of plant growth and morphogenesis, physiological studies suggest many links between PGRs. In fact, several PGRs modulate or are modulated by the ratio of auxin: cytokinin levels and responses. For example, one of the most classical studies in plant physiology is the relation between auxin and cytokinin, which can be employed *in vitro* to induce adventitious buds or roots development, respectively (Skoog and Miller 1957). However, the combination of the synthetic auxin 2,4-D and cytokinin BAP may stimulate, by another mode of action, adventitious buds and somatic embryos formation, as also found by Zhong *et al.* (1992), Nomura and Komamine (1995), and Gaj (2004). In addition, in some plant species, cultures of dedifferentiated cells were initiated in the presence of 1-naphthaleneacetic acid and a few minutes shock of 2,4-D was sufficient to induce somatic embryo formation (Dudits *et al.* 1991). This suggests that 2,4-D has a specific signaling role in the initiation of *de novo* differentiation. Further, 2,4-D application could induce zygotic development in unfertilized maize egg cells, emphasizing its role as a general inducer of the embryogenic response (Kranz *et al.* 1995). However, it is not fully known how and why 2,4-D was effective in the induction of morphogenetic competence, as reported by Pasternak *et al.* (2002).

As previously mentioned, in numerous experimental systems, 2,4-D is commonly used to initiate *de novo* differentiation in tissue cultures of many plant species (Ammirato 1985, Dunstan *et al.* 1995). Not only is the exogenous auxin important in the initiation of adventitious buds and somatic embryogenesis, but changes in endogenous auxin concentrations may also be critical for the developmental stages of regeneration as well (Cooke *et al.* 1993, Krishna-Raj and Vasil 1995). In this study, a higher transient increase in endogenous free IAA concentration occurred in the shoot apex explants as a response to exogenous application of different levels of BAP, with a higher auxin level noted at 5 μM compared to 20 μM BAP, suggesting a positive relationship between the dynamic level of free IAA and type of de novo differentiation (Figs. 1, 2). The data presented in this study are in accordance with those of Rajasekaran *et al.* (1987) Ivanova *et al.* (1994) Michalczuk and Druart (1999), and Jimenez and Bangerth (2001) who reported that higher endogenous IAA concentrations are associated with an increased morphogenetic response. In addition, in carrot cells, exogenous 2,4-D stimulates the accumulation of large amounts of endogenous IAA (Michalczuk *et al.* 1992a, 1992b). These authors hypothesized that the morphogenetic competence of carrot cells is closely associated with a several fold increase in endogenous IAA levels. The timing of the IAA peak noted in this study may be correlated well with the time of the irreversible determination of the differentiation response, as also suggested by Charriére *et al.* (1999). Thus, the present data are in agreement with those obtained by Charriére *et al.* (1999) who reported that the transient increase in the endogenous IAA concentration can be involved in determining the developmental pathway of cultured plant cells.

In addition to free IAA, there is substantial evidence to support the hypothesis that not only free but also conjugated IAA might have biological activity (Kleczkowski and Schell 1995). In the present study, conjugated IAA level was also higher in shoot apices treated with 5 μM than 20 μM BAP (Fig. 2B), indicating that formation of adventitious bud and somatic embryo (Fig. 1) is associated with lower and higher level of conjugated IAA, respectively (Fig. 2). Inhibition of IAA conjugates in shoot apex explants treated with 20 μM BAP might be explained by the fact that this cytokinin at high concentration inhibits free IAA to be conjugated with other molecules, as also reported by Lau and Yang (1973). It is also obvious that the marked increase in the cellular level of conjugated IAA forms has to be preceded by the synthesis of free IAA, as shown in Fig. 2. The physiological roles of IAA conjugates include storage of IAA for subsequent use and protection against degradation, and the conjugates are thought to be a means of IAA transport, possibly including tissue targeting. The equilibrium between free and conjugated IAA is important for the homeostatic control of the concentration of free auxins in the plant. Lastly, conjugates are believed to be involved as a first step in the IAA catabolic pathways (Woodward *et al.* 2005 and references cited therein). Consequently, conjugates play important roles in auxin physiology and metabolism. Based on the forgoing results, it seems likely that higher level of free and conjugated IAA favored inducing somatic embryogenesis, while lower one favored inducing adventitious bud formation (Figs.1, 2).

Unlike the dynamic concentrations of IAA (Fig. 2A), the level of free 2,4-D was almost unchanged during the time-course studied of *de novo* differentiation, as shown in Figure 3, concluding that the natural auxin IAA is eliciting a significant role(s) in plant morphogenesis. However, level of conjugated 2,4-D was significantly higher in 5 μM BAP-treated shoot apex than that in 20 μM one during the early stages of *de novo* differentiation (Fig. 3). Despite unchanging the level of internal 2,4-D in this study, it is still considered to be strong auxin in de novo differentiation culture (Ammirato 1985) because its effects are so pronounced. 2,4-D likes IAA in some aspects: (a) the pool of free 2,4-D was metabolized to form 2,4-D conjugates with glutamic and asparatic acids (Feung *et al.* 1972, Bialek *et al.* 1983); (b) 2,4-D conjugates could also be hydrolyzed back to the free form; and (c) exogenous application

of 2,4-D is also known to influence metabolism of IAA (Michalczuk *et al.* 1992a, b) and formation of auxin conjugates (Sasaki *et al.* 1994). Consequently, conjugated IAA and 2,4-D may have been hydrolyzed in the later stages of regeneration process when high auxin activity is required and/ or conjugated auxins could have been catabolized directly, as also reported by Woodward *et al.* (2005). In addition, one may consider that 2,4-D may act not only as auxin, but also as a stress agent, and thus it may induce stress responses in plant cells, as reported by Grossmann (2000). Early phases of a regeneration process are characterized by the induction of many stress-related genes, which leads to the hypothesis that *de novo* differentiation is an extreme stress response of cultured plant cells, as also suggested by Dudits *et al.* (1995) and Pasternak *et al.* (2002).

Additionally, analytical studies have recently demonstrated that irrespective of the particular auxin, higher levels of free native auxin, IAA, and/ or synthetic auxin, 2,4-D, are invariably associated with early stages of morphogenesis (Michalczuk *et al.* 1992a, Ribnicky *et al.* 1996, Pasternak *et al.* 2002, Gaj 2004). In addition, evidence was provided that auxin plays important roles in morphogenetic pattern formation of monocots and dicots. Investigation of plant morphogenesis by perturbing *in vitro* development of various plant species with exogenous auxin, antiauxin, and auxin transport inhibitors led to the formation of specific abnormal morphogenetic pathways (Schiavone and Cooke 1987, Fischer-Iglesias *et al.* 1997, Hadfi *et al.* 1998). The data presented here are in agreement with those found by Skoog and Miller (1957), Zimmerman (1993), and Mordhorst *et al.* (1997) who reported that adventitious bud formation requires a high cytokinin-to-auxin ratio, and the reverse was true in relation to somatic embryo formation, although analysis of BAP, as well as natural cytokinins, was not performed during the time-course studied.

CONCLUSIONS

The results of the present investigation suggest that exogenous PGRs, auxin and cytokinin, are needed for maize plant morphogenesis. External higher BAP concentration in the presence of low concentration of the synthetic auxin 2,4-D favored the induction of adventitious buds, whereas lower BAP concentration favored inducing somatic embryogenesis. Dynamics of endogenous IAA and 2,4-D concentrations suggests that exogenous application of a lower level of the cytokinin BAP promoted auxin-induced somatic embryogenesis and its higher concentration stimulated another type of regeneration, i.e. bud organogenesis.

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تأثير 2،4 - دايكلوروفينوكسي حمض الخليك و6- بنزيل أمينو بيورين على تكوين البراعم العرضية والأجنة الجسدية للذرة فيما يتعلق بالمستويات الداخلية للأوكسينات الحرة والمرتبطة

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في دراسات سابقة لوحظ أن أجزاء القمم النامية للذرة المنزرعة معمليا يمكن حثها لتكوين إما براعم عرضية أو أجنة جسدية بتغيير محتوى بيئة الزراعة لمنظمات النمو وبوجة خاص الأوكسينات والسيتوكينينات. وكان الهدف من هذا البحث هو دراسة تأثير منظم النمو الأوكسينى المخلق صناعيا 2،4 - دايكلوروفينوكسي حمض الخليك بتركيز 2.5 ميكرومولر فى وجود مستويات مختلفة لمنظم النمو السيتوكينينى 6- بنزيل أمينو بيورين (5، 20 ميكرومولر) على حث القمم النامية للذرة علىالتكوين المورفولوجى للبراعم العرضية أو الأجنة الجسدية عند تنمية تلك القمم فى بيئات زراعة الأنسجة ودراسة العلاقة بين تلك التكوينات المورفولوجية ومحتواها من الأوكسينات (أندول-3-حمض الخليك، 2،4 - دايكلوروفينوكسي حمض الخليك) فى صورتهما الحرة والمرتبطة) أثناء الأحداث الأولية لهذا التكوين المورفولوجي بعد المعاملة. تم فصل القمم النامية من بادرات ذرة عمر 7 أيام تم إنباتها على بيئة زراعة صناعية أولا ثم إعادة زراعة تلك القمم لإكثارها خضريا من ناحية وتجانس التكوين المورفولوجى من ناحية أخرى على نفس بيئات الزراعة ولكنها محتوية على محللات الكازين بتركيز 500 ملليجرام فى اللتر، 6- بنزيل أمينو بيورين بتركيزات 5،20 ميكرومولر مختلط بكل منها المركب الأوكسينى 2،4 - دايكلوروفينوكسي حمض الخليك. تم التقدير الكمى للأوكسينات الحرة والمرتبطة فى العينات النباتية المأخوذة فى توقيتات معينة من نشأة وتطور تلك التكوينات المورفولوجية بإستخدام جهاز التحليل الكروموتوجرافى العالى الأداء. أوضحت النتائج المتحصل عليها أن هناك علاقة بين مسلك القمم النامية المنزرعة معمليا لتكوين البراعم العرضية ومحتواها المنخفض من الأوكسينات الداخلية المشار إليها سابقا وكان العكس صحيحا عند مسلك تلك القمم لتكوين الأجنة الجسدية. وتؤكد النتائج المتحصل عليها أيضا أهمية نسبة الأوكسين إلى السيتوكينين المتواجدة فى بيئة زراعة الأنسجة فى تنظيم تلك التكوينات المورفولوجية. ولقد نوقشت الأدوار الفسيولوجية لكل من السيتوكينين والأوكسين المعامل خارجيا فى كيفية حث القمم النامية على تكوين البراعم العرضية والأجنة الجسدية لما لها من أهمية اقتصادية فى إكثار النباتات خضريا وتحسين انتاجيتها حيث أنة من السهولة بمكان تحوير النباتات وراثيا بإدخال جينات إليها بإستخدام هذا التكنيك من زراعات الأنسجة.