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Effect of thidiazuron on somatic embryogenesis of *Cayratia japonica*

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Abstract

Unpollinated ovary explants of *Cayratia japonica* (Thump.) Gagnep, were cultured on the revised Murashige & Skoog's medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) alone, or in combination with 0.009 μM thidiazuron (TDZ) or 0.23 μM kinetin for induction of embryogenic callus. The best results were obtained on medium containing 2.3 – 4.6 μM 2,4-D and TDZ. When the calluses were subcultured on the basal medium (BM), somatic embryogenesis took place spontaneously at surfaces of the calluses, but only about 5% of the somatic embryos could develop to cotyledonary stage and most of the rest remained at the globular stage of development. If the calluses were transferred onto medium containing TDZ or TDZ combined with 0.27 μM α-napthaleneacetic acid, the number of cotyledonary somatic embryos increased up to 25%. When the somatic embryos of different stages were transferred onto fresh BM, only the cotyledonary embryos could convert into the plantlets. The results revealed that for the induction of embryogenic callus and somatic embryogenesis of *Cayratia japonica*, both cytokinin and auxin are required in the medium and the cytokinin activity of TDZ is much stronger than that of kinetin even when the concentration of TDZ used was only 4% of kinetin.

Abbreviations: BA – benzyladenine, 2,4-D – 2,4-dichlorophenoxyacetic acid, IAA – indole-3-acetic acid, NAA – α-naphthaleneacetic acid, PGR – plant growth regulator, TDZ – thidiazuron (N-phenyl-1,2,3,-thi-diazol-5-ylurea)

Introduction

Cayratia japonia (Thump.) Gapnep. is a perennial herbaceous creeper of the Vitaceae. It is distributed widely in the South and North of China, Japan and the countries of Southeast Asia. In China it has long been used as a popular medicine as an antipyretic, antidote and for subsidence of swelling. Recently it was found to be effective as a bactericide and for curing many diseases (He 1981). We established the in vitro culture of shoots of *Cayratia japonica* that were regenerated from leaf-derived callus (Guo et al. 1989). The regenerated plantlets were transplanted into soil, survived and flowered the next year. In 1990–1991, we cultured unpollinated ovaries and found that TDZ has a significant effect on somatic embryogenesis of *C. japonica*. Herein we report the results about callus induction and somatic embryogenesis in vitro in unpollinated ovaries of *C. japonica*.

Materials and methods

The basal nutrient medium contained the revised Murashige and Skoog's (1962) salts and organic components, 30 g l^{-1} sucrose, 7 g l^{-1} agar (made in China) and supplemented with different concentrations (0, 2.3, 4.6, 6.9 and 9.2 μM) of 2,4-D

alone or in combination with TDZ (0.009 μM) or kinetin (0.23 μM). The media were adjusted to pH 5.7 with 0.1 N NaOH and 30 ml dispensed into each 100 ml Erlenmeyer flask before autoclave sterilization for 20 min at 121°C.

The donor plants were grown in field for two years. Young inflorescences were excised from the plants and then surface disinfected in 70% ethanol for 30 sec and in 0.1% $HgCl_2$ solution for 15 min, then rinsed 3–5 times in sterile water. Afterwards, the perianths and stamens were excised from unopened flowers and the ovaries inoculated on the media, separately and with random orientations. About 20 explants were inoculated into each Erlenmeyer flask with at least three flasks per treatment.

The cultures were maintained at 25–28°C with a 14-h photoperiod.

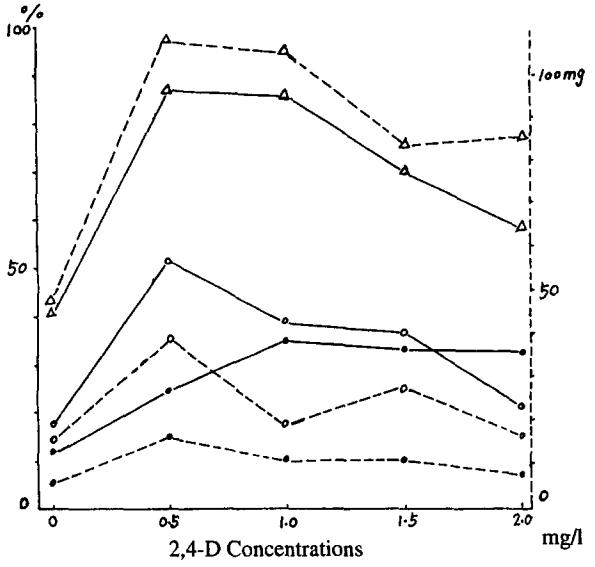

Fig. 1. Callus induction on different media cultured for 5 weeks. 2,4-D alone ●; 2,4-D and kinetin (0.23 μM) ○; 2,4-D and TDZ (0.009 μM) △. ——— for the callus induction frequencies (%), – – – – – for the callus fresh weight per explant (mg).

Results

Induction of callus

Figure 1 shows that some of the explants on the basal medium (BM) could give rise to callus, although both the induction frequency and fresh weight of callus per explant were very low (6% and 6 mg, respectively). On media supplemented with 2.3–9.2 μM 2,4-D, the callus induction frequencies and callus fresh weight per explant increased to 25–35% and 8–16 mg, respectively (Fig. 2a). If the media contained 0.23 μM kinetin or 0.009 μM TDZ only, it could induce callus production also, and induction frequencies and fresh weight of calluses were higher than those on BM and the media containing 2,4-D only. At the same time, TDZ is much more active than kinetin. The induction frequencies and fresh weight of callus per explant on the media containing TDZ were obviously higher than those on the media with kinetin, even though the concentration of TDZ used is much lower than that of kinetin (Fig. 1).

When media contained 2,4-D and TDZ or kinetin, the two kinds of PGR acted synergistically on callus induction and proliferation of callus of *C. japonica*. On media containing TDZ and different concentrations of 2,4-D, the induction frequencies and fresh weight of callus per explant were the highest when 2,4-D was 2.3 μM. When the concentrations of 2,4-D were 4.6 μM or higher, the values decreased a little, but still maintained at levels 2 to 3 times higher than those on media supplemented with 2,4-D alone. But when 2,4-D was combined with kinetin, only on medium with 2.3 μM 2,4-D was the synergism of the two PGRs displayed and it was obviously lower than that of TDZ and 2,4-D. When concentrations of 2,4-D were higher than this, the synergism decreased or even disappeared (Fig. 1 and 2b, c).

Somatic embryogenesis

The calluses induced on different media were white, fragile and similar in colour and surface structure. When the calluses were subcultured on BM, only the calluses that were obtained from media containing 2.3–4.6 μM 2,4-D combined with TDZ or kinetin and were growing well could give rise to somatic embryogenesis. The other calluses, especially those obtained on media containing only one PGR (2,4-D, TDZ or kinetin), grew poorly and did not produce any

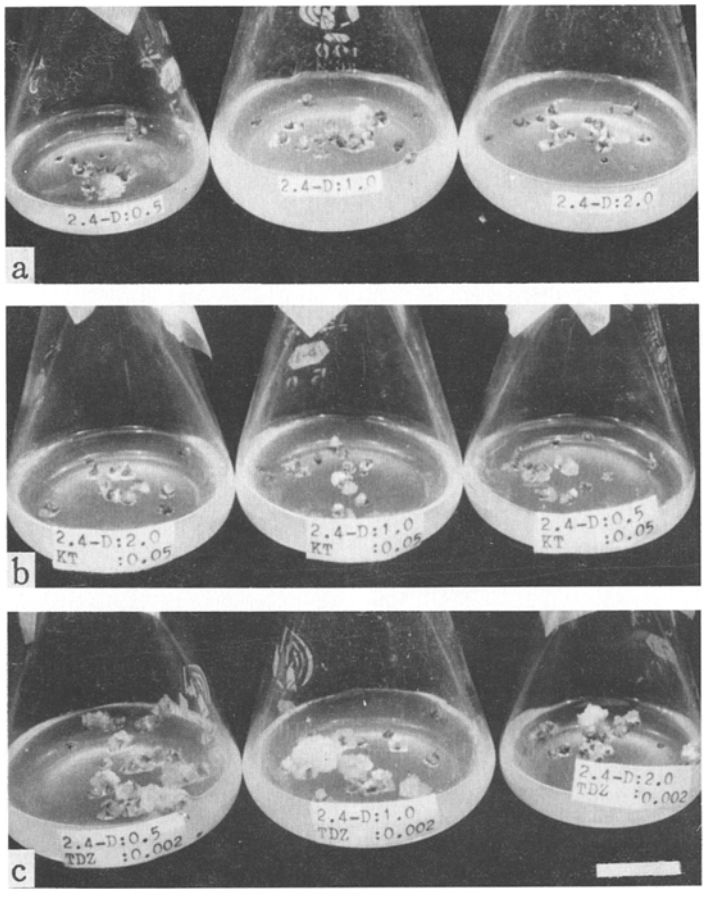

Fig. 2. Callus induction on different media cultured for 5 weeks (bar = 2.0 cm). (*a*) On medium containing 2,4-D only; concentrations of 2,4-D are 2.3, 4.6 & 9.2 μM (0.5, 1.0, 2.0 mg l^{-1}) respectively. (*b*) On medium containing 0.23 μM (0.05 mg l^{-1}) kinetin and 2,4-D of the same concentrations as above. (*c*) On medium containing 0.009 μM (0.002 mg l^{-1}) TDZ and 2,4-D of the same concentrations as above.

somatic embryos. Therefore, only the former were embryogenic calluses. In one to two weeks of subculture, many globular somatic embryos appeared on the surface of the embryogenic callus. After five weeks of culture, some of these embryos developed into cotyledonary ones (Fig. 3a). On the basal medium only about 5% of somatic embryos developed into cotyledonary stage and few (no more than 1%) converted into plantlets. Most of the embryos remained at the globular, heartshaped or torpedo stage. Many of the torpedo-stage embryos became abnormal trumpet or vase-shaped ones (Fig. 3b, c, d). On the media supplemented with one of the cytokinins (0.009 μM TDZ or 0.23 μM kinetin) alone or combined with an auxin (NAA 0.27 μM), callus proliferation and somatic embryogenesis could be greatly influenced by the PGR used in the medium. The percentages of cotyledonary embryos increased to about 10% on medium containing kinetin combined with NAA and 25% on medium containing TDZ combined with NAA. The growth of somatic embryos on the latter medium was obviously better than that on the former one (Fig. 3e, f).

Conversion of somatic embryos into plantlets

After transfer of somatic embryos of different stages onto the fresh BM and culture for five weeks, a majority of the cotyledonary embryos (above 90%) converted into plantlets, although on the original BM, only a few of them completed this conversion (Fig. 3d, g). At the same

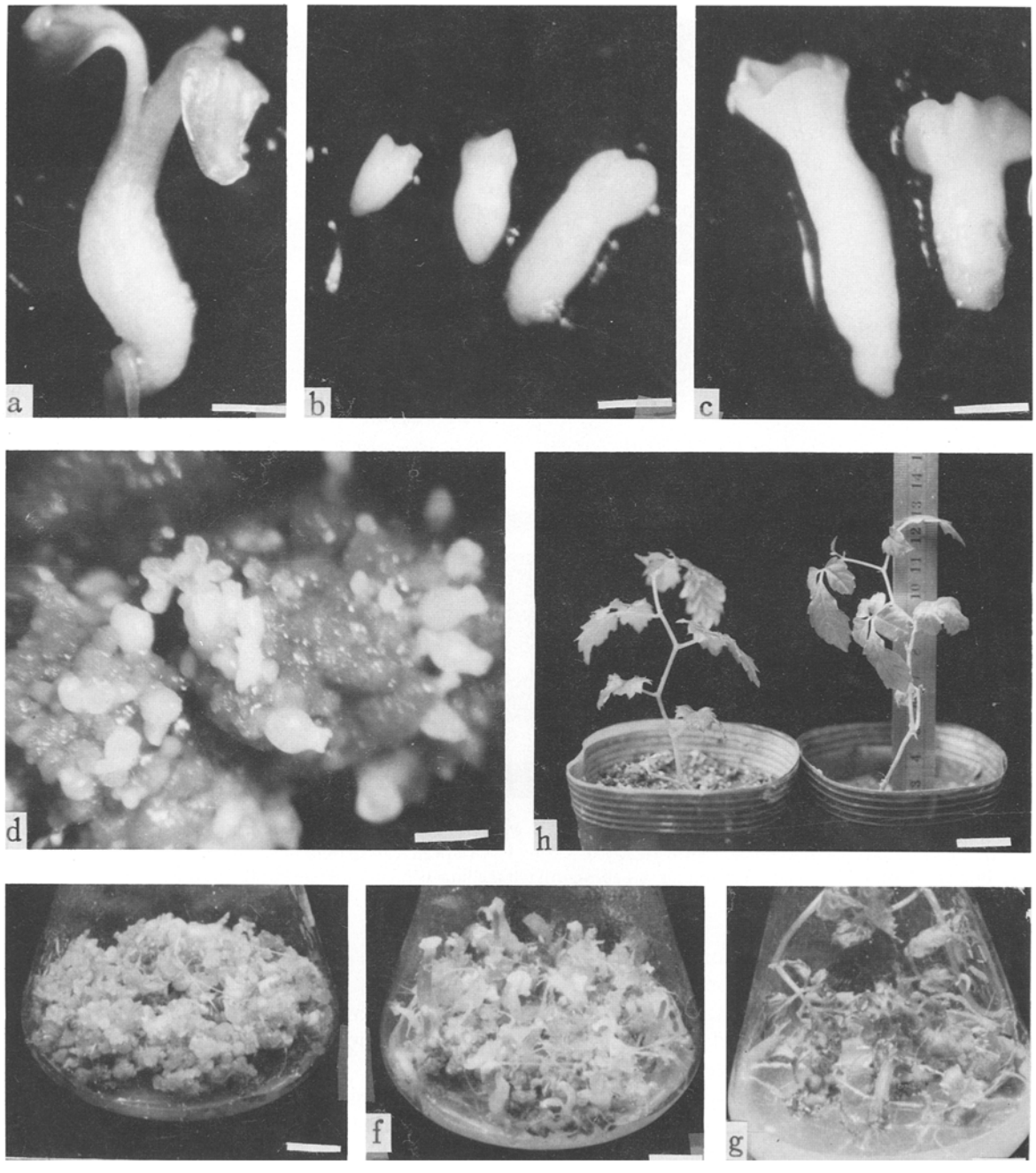

Fig. 3. Somatic embryogenesis and plant conversion. (*a*) A cotyledonary somatic embryo (bar = 0.2 cm). (*b*) Heart-shaped and torpedo somatic embryos (bar = 0.2 cm). (*c*) Abnormal trumpet-shaped somatic embryos. (bar = 0.2 cm). (*d*) Different stages of somatic embryos at the surface of embryogenic callus subcultured on BM for 3–4 weeks, some of the embryos appeared vase-shaped ones. (bar = 0.1 cm). (*e*) Somatic embryogenesis on medium containing 0.23 μM (0.05 mg l^{-1}) kinetin & 0.27 μM (0.05 mg l^{-1}) NAA (bar = 1.0 cm). (*f*) Somatic embryogenesis on medium containing 0.009 μM (0.002 mg l^{-1}) TDZ & 0.27 μM (005 mg l^{-1}) NAA (bar = 1.0 cm). (*g*) Plantlets coverted from the cotyledonary somatic embryos on the fresh BM (bar = 1.0 cm). (*h*) Plants in pots (bar = 1.0 cm).

time, the embryos that remained in the earlier stages, particularly the morphologically abnormal embryos, could not complete their development and conversion after they were transferred onto fresh BM.

The plantlets converted from somatic embryos on fresh BM in culture of five weeks were about 2.0 cm tall or taller, with one or two leaves above the small cotyledons (Fig. 3g). The plantlets have been transplanted into soil and survived (Fig. 3h).

Discussion

The earliest work on somatic embryogenesis demonstrated that for many plants, e.g. *Daucus carota* (Halperin & Wetherell 1964), *Oryza sativa* (Bajaj & Bidani 1980), *Triticum aestivum* (Ozias-Akins & Vasil 1982), and *Zea mays* (Lu et al. 1982), induction of embryogenic callus required the presence of an auxin in the medium. 2,4-D is the most effective and commonly used one. When the callus was subcultured on a basal medium or a medium containing 2,4-D or other auxins at much lower concentrations than in the medium for induction of callus, somatic embryogenesis can spontaneously take place in the callus (Ammirato 1983). That means that the entire process of initiation and development of somatic embryos of these plants can be induced and completed by one kind of PGR. At the same time, some other plants, e.g. *Apium graveolens* (Williams & Collin 1976), and *Lycopersicon peruvianum* (Zapata & Sink 1981) required the presence of both auxin and cytokinin in the medium for somatic embryogenesis in vitro. According to the reference survey of growth regulator usage for inducing embryogenesis made by George & Sherrington (1984), cytokinins were used in the primary culture (to induce embryogenic callus) for 55% of broad-leafed species and 32% of grass and cereal species. In the secondary culture (for somatic embryogenesis), cytokinins were used in 32% of broad-leafed species and 24% of grass and cereal species. Recent studies have revealed that more and more plants, including dicotyledons, monocotyledons and gymnosperms, e.g. *Populus ciliata* (Cheema 1989); *Eleusine coracana* (George & Eapen 1990) and *Pinus caribaea* (Laine & David 1990), should be put into the latter category. Therefore, the action of cytokinin in somatic embryogenesis is very important. Even in wild carrot (*Daucus carota*), which does not require cytokinin in the medium for somatic embryogenesis, Durley et al. (1984) demonstrated that in the embryogenic cell line WOO1C, the amount of endogeneous cytokinins increased significantly when the cells were transferred from medium containing 2,4-D to one without it, at the same time, the level of IAA did not change. But in non-embryogenic cell line WOO1, the level of IAA increased while cytokinin amount was unchanged. Hanower & Hanower (1984) indicated also that in oil palm (*Elaeis guineensis*), when the clones of embryogenic cells were treated with 8-aza-guanine, a cytokinin-antagonist, somatic embryogenesis was inhibited. However, when they were treated with anti-auxins, e.g. 2-0-chlorophenoisobutyric acid and 7-aza-indole, somatic embryogenesis was stimulated, so it was suggested that a high level of endogenous cytokinins was necessary for embryogenesis to proceed. Furthermore, Southworth & Kwiatkowski (1991) with *Limnanthes alba* and Kiss et al. (1992) with *Aesculus hippocastanum* demonstrated that cytokinins in combination with auxin significantly stimulated somatic embryogenesis from immature embryos, and that secondary somatic embryogenesis, both in numbers and size of adventive somatic embryos, was directly related to the concentration of BA used in medium. In *Ceratozamia hildae*, *Zamia furfuracea* and *Z. pumila*, Chavez et al. (1992a, b) found that, embryogenic calluses were induced from immature embryos in the presence of cytokinin alone. Dudits et al. (1991) indicated that as a routine procedure, the synthetic auxins, typically 2,4-D, are the key factors for induction of osmatic embryogenesis, although 'cytokinin can also be an optional component of the culture medium'. Our experiment showed that for somatic embryogenesis of *Cayratia japonica*, cytokinin presence in medium and its synergism with auxin were very important both in induction of callus and initiation and development of somatic embryos. From our results and those of other authors, however, we actually do not know what is the exact action of cytokinins on somatic

embryogenesis. It should be carefully studied further.

Thidiazuron and several substituted pyridyl phenyl urea compounds have been demonstrated to stimulate meristem and shoot formation in vitro at unusually low concentrations (Fellman et al. 1987). These compounds appear to have a strong cytokinin-like effect in a wide range of species, and even in the species that respond little to conventional, adenine-based cytokinins, although the compounds have molecular structures that are very different from those of adenine-based cytokinins (Reynolds 1987; Zhou & Guo 1990). Thidiazuron has displayed the properties of stimulating callus induction and growth, breaking bud dormancy, promoting bud proliferation and inducing organogenesis in vitro in many plants, e.g. *Carica pentagona* (Zhou & Collet 1989), *Malus domestica* (Wang et al. 1986; Van Nieuwkerk et al. 1987) and *Rubus* sp. (Fiola et al. 1990). Yip & Yang (1984) showed that TDZ can act synergistically with IAA and Ca^{2+} just as do the adenine-based cytokinins, e.g. BA (Lau & Yang 1973).

Recently, Saxena et al. (1992) first reported that somatic embryogenesis was induced by TDZ alone from intact seedlings of peanut (*Arachis hypogaea*). These authors hypothesized that 'TDZ helps to establish within the developing seedlings the optimum balance of cytokinin: auxin required for somatic embryogenesis.' Therefore, they emphased that the structural or morphological integrity of seedlings is related to their response to TDZ. In *Cayratia japonica*, TDZ showed cytokinin activity and synergism with auxins (2,4-D or NAA) in both induction of embryogenic callus and somatic embryogenesis in ovary explants. The activity of TDZ was much stronger than that of kinetin even though the concentration of TDZ (0.009 μM) used in the experiment was only about 4% of that of kinetin (0.23 μM). These results are very similar to the reports on the application of TDZ in organogenesis in vitro in many plants (Zhou & Guo 1990).

Although somatic embryogenesis of *C. japonica* could be induced in media supplemented with 2,4-D in combination with TDZ or kinetin, the percentage of cotyledonary embryos in BM was relatively low (about 5%). TDZ and kinetin promoted the development of somatic embryos and increased the number of cotyledonary embryos to 10–25%, but most of the other embryos did not develop further even on media containing TDZ or TDZ in combination with NAA. At the same time, a number of somatic embryos of *C. japonica* became morphologically abnormal as in soybean (Ranch et al. 1986). These abnormal embryos never successfully converted into whole plantlets regardless of the subsequent media. Further studies, therefore, are necessary for development of somatic embryos in *C. japonica*. For example, addition of other plant growth regulators or changes in physical conditions of cultures, as in *Apium graveolens* (Nadal et al. 1990), *Glycine soja* (Ranch et al. 1986) and *Triticum aestivum* (Carman 1988), can be expected to improve the development of somatic embryos in *C. japonica*.

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