

Note

Anise-cultured cells abolish 2,4-dichlorophenoxyacetic acid in culture medium

Kumi Tanida, Hajime Shiota*

Graduate School of Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama, Kanagawa 236-0027, Japan

*E-mail: hshiota@yokohama-cu.ac.jp Tel & Fax: +81-45-787-2318

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Abstract In anise (*Pimpinella anisum*, family Apiaceae), callus-like embryogenic cells (embryogenic callus) are induced by culturing hypocotyl explants in 2,4-dichlorophenoxyacetic acid (2,4-D)-containing medium, and somatic embryos are formed from embryogenic callus transferred into 2,4-D-free medium. Anise somatic embryos are also induced even if embryogenic callus is continually cultured in 2,4-D-containing medium without subculturing. In this study, we aimed to clarify the dynamics of 2,4-D during anise cell culture. After culturing anise callus in 2,4-D-containing medium, 2,4-D in the medium was analyzed by thin-layer chromatography. In the medium, 2,4-D was decreased during anise callus culture, and fully abolished after 5-day culture. On the other hand, no decrease in 2,4-D was observed in the other Apiaceae species (carrot, fennel, dill, parsley, and coriander). After 7-day culture of anise callus, the medium was collected following removal of the cultured cells and 2,4-D was added to the collected medium. After 10 days of incubation and shaking, 2,4-D was markedly decreased in the medium. However, when the collected medium was heat-treated at 100°C, 2,4-D was detected after 20 days of incubation. Therefore, anise callus has a specific 2,4-D degradation system, in which heat-inactivated secreted molecules may participate.

Key words: anise, callus, 2,4-dichlorophenoxyacetic acid, thin-layer chromatography, tissue culture.

Carrot (*Daucus carota*, family Apiaceae) somatic embryogenesis has been used as an experimental model of plant embryogenesis (Mahdavi-Darvari et al. 2015). In carrot, callus-like embryogenic cells (embryogenic callus) are induced from hypocotyl explants in Murashige and Skoog (MS) medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), an auxinic herbicide (Ikeda et al. 2006). Carrot somatic embryos are formed from embryogenic cells transferred into 2,4-D-free medium, while callus-like cell clusters are maintained in the presence of 2,4-D (Ikeda et al. 2006). In some Apiaceae species, such as fennel (*Foeniculum vulgare*), anise (*Pimpinella anisum*), coriander (*Coriandrum sativum*) and celery (*Apium gargeolens*), somatic embryogenesis has been reported using similar culture methods (Huber et al. 1978; Maheshwari and Gupta 1965; Nadel et al. 1989; Zee 1981).

In anise, an outbreeding species whose fruit (aniseed) is used as a culinary spice, tissue culture systems have been developed to yield a heritably uniform cultivation line (Bela and Shetty 1996). Anise embryogenic callus is induced from root explants by culturing in 2,4-D-containing medium for 30–40 days, and

somatic embryos are formed from embryogenic cells transferred into 2,4-D-free medium (Bela and Shetty 1996, 1999; Huber et al. 1978). Anise somatic embryos are also formed when embryogenic callus is cultured in 2,4-D-containing medium for 30–40 days without subculturing (Bela and Shetty 1996). Therefore, it is assumed that 2,4-D is degraded in medium during anise cell culture, or that anise cells decrease in terms of their sensitivity to 2,4-D.

More than 32 kinds of 2,4-D-resistant plants are known (Quareshy et al. 2018), and numerous approaches to counteract 2,4-D have been proposed, including side-chain degradation or extension, ring hydroxylation, sugar conjugation, amino acid conjugation, reduction of uptake or translocation, and specific efflux (Eyer et al. 2016; Figueiredo et al. 2018; Hamburg et al. 2001; Ito and Gray 2006; Quareshy et al. 2018; Rey-Caballero et al. 2016). The mechanisms of metabolism and perception of 2,4-D in anise cells remain unclear. In the present study, we aimed to clarify the dynamics of 2,4-D during anise cell culture.

Seeds of six Apiaceae species were used: anise (*Pimpinella anisum*), carrot (*Daucus carota*), fennel

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; MS medium, Murashige and Skoog medium; MSD medium, 2,4-D-containing MS medium; Rf, retardation factor; TLC, thin-layer chromatography; UV, ultraviolet.

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(*Foeniculum vulgare*), dill (*Anethum graveolens*), parsley (*Petroselinum crispum*), and coriander (*Coriandrum sativum*) (Mikasa Engei, Tokyo, Japan; Yokohama Nursery, Yokohama, Japan). Seedlings were grown on vermiculite for 10–14 days at 25°C with 16 h of light (approximately 40 mmol photons m⁻² s⁻¹) daily, and sterilized with 1% sodium hypochlorite. To induce callus, 10-mm lengths of hypocotyls were cultured on 2,4-D (1 mg l⁻¹)-containing MS (MSD) semi-solid medium at 25°C in the dark and subcultured every 2 weeks (Bela and Shetty 1996; Ikeda et al. 2006). The callus was cultured in 100 ml of MSD liquid medium by shaking (100 rpm), and subcultured every 2 weeks. In all six Apiaceae species, calli were induced in MSD medium for 2–3 months (Supplementary Figure S1). In anise, somatic embryos, buds, and roots were formed when callus was cultured in MSD medium for more than 2 weeks without subculturing (Supplementary Figure S2). The redifferentiation of anise callus in 2,4-D-containing medium is supported by Bela and Shetty (1996). On the other hand, somatic embryos, buds, and roots were not observed in carrot, fennel, dill, parsley, or coriander after continuous callus culture in MSD medium. Thus, the redifferentiation in 2,4-D-containing medium was observed specifically in anise.

The 2,4-D-containing samples and MSD liquid medium were adjusted to pH 2.0 with 6N H₂SO₄ (Feung et al. 1978; Guardigli et al. 1971). An equivalent volume of diethyl ether was added to the medium, which was then mixed, and the diethyl ether layer collected. The concentrated diethyl ether layer was separated by thin-layer chromatography (TLC) (250 μm silica gel layer; Whatman 4420 222, GE Healthcare, Buckinghamshire, UK) using 100% ethanol. Separated 2,4-D was detected under ultraviolet (UV) light (254, 302 and 365 nm) and appeared as a blackish spot (Rf=0.59) (Figure 1; see Feung et al. 1978). The UV-absorbable spot observed in MSD medium (Rf=0.81) was presumed to be derived from MS medium, because it was not detected in the 2,4-D-containing sample (Figure 1).

The calli (approximately 0.5 g) were cultured in 100 ml of MSD liquid medium for 7 and 14 days in the dark at 25°C. They grew well and their weight increased approximately 2.5-fold for 7 days. The cultured cells were removed from the cultures by centrifugation (4,200×g for 5 min) and filtration (pore size <5 μm; Advantec No. 2, Advantec, Tokyo, Japan), and the separated media were then collected. The diethyl ether extracts from the collected media were analyzed by TLC. The 2,4-D was not detected in the medium in which anise callus had been cultured for 7 or 14 days (Figure 2A). On the other hand, 2,4-D was detected in the media in which calli of carrot, fennel, dill, parsley, and coriander had been cultured for 7 days (Figure 2A, B). Thus, the decrease in 2,4-D in MSD medium was specific to anise among the

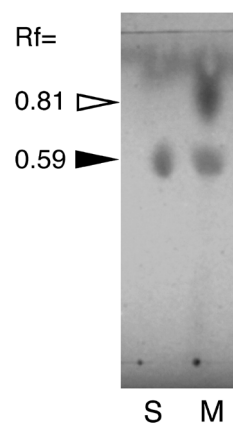


Figure 1. Detection of 2,4-D by thin-layer chromatography. The diethyl ether extracts from 2,4-D-containing samples (S) and 2,4-D-containing MS (MSD) liquid medium (M) were separated by TLC and detected under ultraviolet (UV) illumination. Black and white arrowheads show the positions of 2,4-D and the MS medium ingredients, respectively.

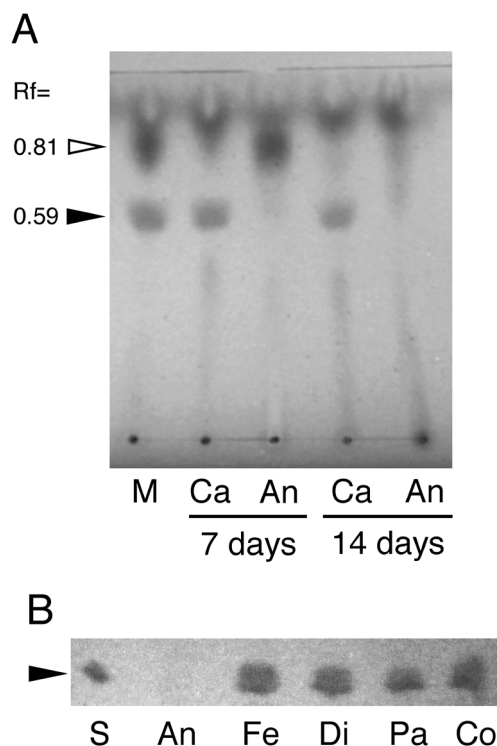


Figure 2. Detection of 2,4-D in Apiaceae callus cultures. The diethyl ether extracts from MSD liquid medium after callus culture were analyzed by TLC. (A) Calli of carrot (Ca) and anise (An) were cultured for 7 or 14 days. (B) Calli of anise (An), fennel (Fe), dill (Di), parsley (Pa), and coriander (Co) were cultured for 7 days. Black and white arrowheads show the positions of 2,4-D and the MS medium ingredients, respectively. S: 2,4-D-containing sample, M: MSD medium.

Apiaceae species examined.

The UV-absorbable spots of putative 2,4-D metabolites were not detected by TLC (Figure 2A). This indicates that 2,4-D may have changed into materials that were insoluble in diethyl ether, or that UV absorbency of

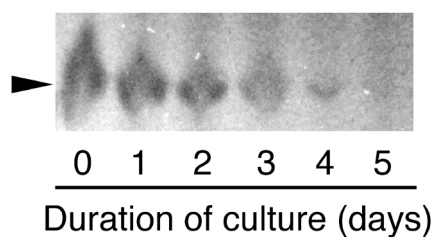


Figure 3. Detection of 2,4-D during anise callus culture. The diethyl ether extracts from MSD liquid media after culturing anise callus for 1–5 days were analyzed by TLC. The black arrowhead shows the position of 2,4-D.

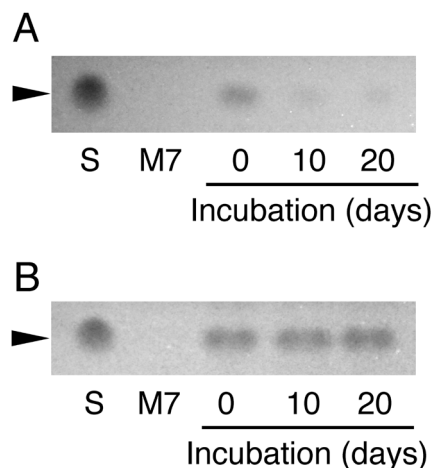


Figure 4. Detection of 2,4-D in medium collected after anise callus culture. Anise callus was cultured in MSD liquid medium for 7 days. The cultured cells were removed from the culture and the medium was collected (M7). Then, 2,4-D was added to the collected medium with no treatment (A) or heat-treatment (B), and incubated by shaking. After 0, 10, and 20 days of incubation, the diethyl ether extractions from the media were analyzed by TLC. Black arrowheads show the positions of 2,4-D. S: 2,4-D-containing sample.

2,4-D was lost by degradation of the ring structure. In several plant species, it has been reported that 2,4-D is changed to conjugate form with sugars or amino acids (Figueiredo et al. 2018; Quareshy et al. 2018; Rey-Caballero et al. 2016). Because sugar conjugates, but not amino acid conjugates, of 2,4-D do not readily dissolve in organic solvents (Davidonis et al. 1980; Feung et al. 1978; Hamburg et al. 2001), we could not extract them to diethyl ether.

The anise callus was cultured in MSD liquid medium for 1–5 days. The spots of 2,4-D became fainter with time in culture (Figure 3), and were almost undetectable in the 5-day culture (Figure 3). In this way, a time-dependent decrease in 2,4-D in MSD medium during anise callus culture was confirmed. The experiments using different cell lines provided similar results, although the rate of decrease in 2,4-D differed among cell lines (data not shown).

The MSD liquid medium in which anise callus had been cultured for 7 days, and 2,4-D was abolished, was

collected after removing the cells (Figure 4). Then, 2,4-D was re-added to the collected medium at 1 mg l^{-1} of the final concentration. The medium with added 2,4-D was incubated by shaking (100 rpm) at 25°C in darkness. The diethyl ether extracted from the incubated medium was analyzed by TLC using benzene–acetic acid (75:12, v/v) (Guardigli et al. 1971). A marked decrease in 2,4-D was observed in the diethyl ether extracted from the media incubated for both 10 and 20 days (Figure 4A). This indicates that 2,4-D decreased even after anise cells were removed. On the other hand, when the collected medium was heat-treated at 100°C for 30 min, the added 2,4-D did not decrease after incubation for 10 and 20 days (Figure 4B). These results suggest that the decrease in 2,4-D may be due to molecules (i.e., putative proteins) secreted from anise cells and inactivated at high temperatures. In plants, amido synthase, GRETCHEN HAGEN 3 proteins and cytochrome P450 have proposed as the enzymes responsible for degradation of 2,4-D (Chiu et al. 2018; Eyer et al. 2016; Figueiredo et al. 2018). Meanwhile, in anise cell cultures, glyoxisomes develop and higher activities of isocitrate lyase and malate synthase (glyoxysomal enzymes) are observed (Kudielka and Theimer 1983). The secreted molecules responsible for the decrease in 2,4-D are assumed to have functions similar to those of the above enzymes.

When anise callus was continually cultured without subculturing, 2,4-D decreased and was eventually abolished. The decrease in 2,4-D may therefore be specific to anise callus. The decrease occurs extracellularly and secreted molecules are likely to participate in it. Our results may lead to the development of technology to detoxify 2,4-D, allowing it to be used as a herbicide in conjunction with anise cells. Analyses using high-performance liquid chromatography and gas chromatography-mass spectrometry, or tracer experiments using $[^{14}\text{C}]$ -2,4-D, are necessary to detect 2,4-D and 2,4-D metabolites more precisely. Furthermore, we plan to identify the enzymes responsible for the breakdown of 2,4-D in further research using different tissues and cell lines.

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