Phytochemistry Letters 7 (2014) 38–41

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Effect of nicotinic acid, nicotinamide and trigonelline on the proliferation of lettuce cells derived from protoplasts

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ARTICLE INFO

Article history:
Received 15 July 2013
Received in revised form 11 September 2013
Accepted 13 September 2013
Available online 8 October 2013

Keywords:
Lactuca sativa
Lettuce
Protoplast
Nicotinic acid
Trigonelline
Physiological function
Detoxification

ABSTRACT

To investigate the physiological role of trigonelline in plant cells, the effects of nicotinic acid, nicotinamide and trigonelline on the division and colony formation of lettuce cells were investigated. Four days after treatment with 0.1–1.0 mM nicotinic acid, division of isolated protoplasts was significantly inhibited. In contrast, a little or no inhibition was found in protoplasts treated with nicotinamide or trigonelline. Nine days after treatment there was a marked inhibitory effect on the colony formation of cells derived from the protoplasts treated with nicotinic acid or nicotinamide, but no effect or even a stimulatory effect was observed in trigonelline-treated protoplasts. These observations imply that nicotinic acid is toxic in high concentration for cell division of plant cells. Trigonelline formation from nicotinic acid and nicotinamide appears to be a result of detoxification of nicotinic acid which is produced by the pyridine nucleotide cycle in the cells or supplied exogenously to the cells.

1. Introduction

Nicotinic acid, nicotinamide and trigonelline (Fig. 1) are pyridine compounds derived from pyridine nucleotides. In plants, nicotinamide is formed as a catabolite of NAD and NADP, and is converted to nicotinic acid by plant specific nicotinamidase (EC 3.5.1.19) (Zrenner and Ashihara, 2011). Some nicotinic acid is converted to nicotinic acid mononucleotide in a reaction catalyzed by a pyridine salvage enzyme, ATP-dependent nicotinate phosphoribosyltransferase (EC 2.4.2.11), and is used for NAD synthesis. In many plants, nicotinic acid is converted to trigonelline (*N*-methyl nicotinic acid) by trigonelline synthase (EC 2.1.1.7). Trigonelline is accumulated in many plant seeds, with especially high content in legumes (Matsui et al., 2007; Tramontano et al., 1986). Small amounts of trigonelline have been also found in aerial parts of many angiosperm plants (96 of the 143 species examined) (Blunden et al., 2005).

Trigonelline in seeds may act as a form of storage of nicotinic acid, although trigonelline demethylase, which catalyses the conversion of trigonelline to nicotinic acid, is not always present in sufficient amounts in plant seeds (Zheng et al., 2005). Marked catabolism of trigonelline is found in roots only in symbiotic conditions with leguminous bacteria, such as *Rhizobium meliloti* (Boivin et al., 1990).

As well as its role in nutritional nitrogen storage, trigonelline may have special functions as a regulator. It is well known that the flavonoids exuded by many legumes act as signals to their rhizobial symbionts. In alfalfa (*Medicago sativa*), trigonelline released into the rhizosphere specifically activates the expression in *Rhizobium meliloti* of a class of genes (*trc* genes) that are involved in trigonelline catabolism (Boivin et al., 1990). Ueda et al. (1995) reported that trigonelline was isolated from *Aeschynomene indica* as a bioactive substance for nyctinasty. Low concentrations (~0.1 μM) were reasonably effective at inducing leaf closing of this species in the daytime. It has also been proposed that trigonelline formation is a response to oxidative, UV and salt stresses (Berglund et al., 1996; Tramontano and Jouve, 1997).

Related to cell division, Evans et al. (1979) identified trigonelline as the substance in *Pisum sativum* cotyledons that promotes G2 arrest in root and shoot meristems. Mazzuca et al. (2000) reported that a high concentration (3 mM) of trigonelline caused cell arrest

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http://dx.doi.org/10.1016/j.phytol.2013.09.008

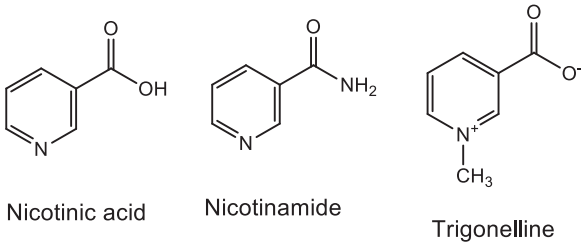

Fig. 1. Structures of nicotinic acid, nicotinamide and trigonelline.

in G2, and prevented ligation of replicons in the S-phase in lettuce root meristems.

In our studies of the metabolism of pyridine nucleotides, we often found that large amounts of [^{14}C]trigonelline were produced when [carbonyl-^{14}C]nicotinamide or [carboxyl-^{14}C]nicotinic acid was administered to plant tissue segments (Ashihara et al., 2010; Matsui et al., 2007). To clarify the role of trigonelline formation at cellular level, we examined the effect of these three pyridine compounds on the proliferation of cells produced from a single lettuce protoplast. We found that greater than 100 μM nicotinic acid and nicotinamide inhibited the division and colony formation of lettuce cells. The role *in Planta* of trigonelline formation is discussed in detail below.

2. Results and discussion

2.1. Proliferation of lettuce protoplasts

To investigate the effect of pyridine compounds on the proliferation of cells, we chose a currently established unique lettuce protoplast culture system, which was originally designed to investigate the allelopathy (Sasamoto et al., 2013). By using this system, it was easy to count the numbers of cells or colonies at different stages of proliferation under an inverted microscope.

Typically, protoplasts were isolated from leaves of 11-day-old seedlings (stage A, Fig. 2). Cell wall formation was observed in isolated protoplasts by 24 h after culture. The non-spherical enlarged cells (stage B) and divided cells (stage C) appeared two days after isolation of protoplasts. Further cell division then took place, and a colony was observed (stage D). At day 4, we counted the numbers of enlarged and divided cells; the mean and SE were 42 ± 5. Most of the cells counted were enlarged cells (stage B), and only 2% were divided cells (stage C). At day 9 there were many divided cells and various colonies (stages C and D). There were 21 ± 2 colonies of 2–3 cells, and 15 ± 2 colonies of more than 4 cells.

2.2. Effect of pyridine compounds on protoplast proliferation

The effect of nicotinic acid, nicotinamide and trigonelline on the proliferation of lettuce cells was investigated. The total numbers of cells of stage B and stage C at day 4 were significantly reduced by 100 and 1000 μM nicotinic acid. In contrast, a little or no effect was found using nicotinamide and trigonelline (Fig. 3A). Nine days after culture, the number of colonies consisting of more than 4 cells was reduced by the addition of more than 100 μM nicotinic acid and nicotinamide, but trigonelline gave no significant effect (Fig. 3B).

The results indicate that a high concentration of nicotinic acid is a potent inhibitor of cell proliferation in the short culture period (4 days), whereas nicotinic acid and nicotinamide both inhibited growth over the longer culture period (9 days). In contrast, trigonelline, up to 1 mM, has no effect on proliferation up to the end of culture.

2.3. Physiological significance of trigonelline synthesis

In many organisms, nicotinamide and nicotinic acid, which are degradation products of NAD and NADP, are re-utilized for the synthesis of pyridine nucleotides by salvage pathways. The route consists of several reactions involved in the degradation and re-synthesis of pyridine nucleotides has been referred to as the pyridine nucleotide cycle (Gholson, 1966; Waller et al., 1966). A typical pyridine nucleotide cycle operating in plant cells is shown in Fig. 4. Unlike animals (Revollo et al., 2004; van der Veer et al., 2007), plants readily convert nicotinamide to nicotinic acid with plant-specific nicotinamidase (Ashihara et al., 2005; Wang and Pichersky, 2007), and salvage nicotinic acid for nicotinic acid mononucleotide synthesis. The most likely rate-limiting step of the pyridine nucleotide cycle appears to be the step catalyzed by nicotinate phosphoribosyltransferase. This enzyme requires ATP for its activity (Zheng et al., 2005). As a result, availability of ATP as well as 5-phosphoribosyl-1-pyrophosphate regulates the formation of nicotinic acid mononucleotide.

In some circumstances, the formation of nicotinic acid mononucleotide from the pyridine nucleotide cycle is discontinued, and nicotinic acid is accumulated. The role of trigonelline formation therefore appears to be detoxification of nicotinic acid, a harmful compound which is formed in plants by the pyridine nucleotide cycle. Nicotinamide also had a similar inhibitory effect

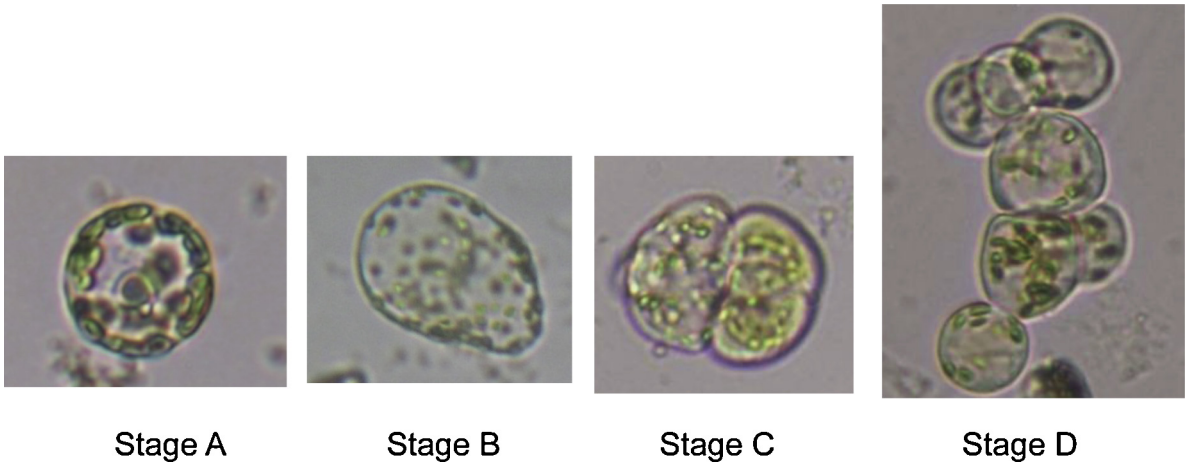

Fig. 2. Proliferation of lettuce cells derived from a single protoplast. Shapes of cells of four typical growth stages are shown. Stage A: an isolated protoplast; stage B: a non-spherically enlarged cell; stage C: divided cells; stage D: a colony with more than 4 cells.