SIMPLE BIOASSAY FOR MEASURING INHIBITORS OF THE 2-*C*-methyl-*D*-erythritol 4-phosphate (MEP) PATHWAY

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ABSTRACT: The 2-*C*-methyl-*D*-erythritol 4-phosphate (MEP) pathway leading to the synthesis of isopentenyl-phosphate (IPP) in plastids has a number of important steps that could be used as new herbicide target sites. The activity of the enzymes of this pathway is very difficult to measure *in vivo* and require cloning of individual genes and their heterologous expression for *in vitro* analysis. We have developed a simple leaf discs bioassay that can identify inhibitors of the early steps in the carotenoid biosynthesis pathway. The level of phytoene accumulation is a measure of the carbon flow in this pathway, and any compound reducing the level of phytoene accumulation is likely to interfere with one of the steps in the MEP pathway. To test this concept, we used known inhibitors of steps of the pathway. It was concluded that this assay may enable the rapid screen of new inhibitors of the MEP pathway in plants.

Keywords: nonmevalonate pathway, inhibitors, herbicide, phytoene, mode of action

INTRODUCTION

In the literature, isoprenoid, terpenoid, and terpene are used interchangeably to refer to a class of natural products essential in all living organisms. Carotenoids comprise a large isoprenoid family and in plants, besides other functions, participate in photosynthetic processes (VRANOVÁ et al., 2013; DELLAPENNA; POGSON, 2006).

Structurally, carotenoids are derived from the 5-carbon units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Through evolution, two non-related and independent biosynthetic routes have been selected for the synthesis of these two basic building blocks (LICHTHENTALER, 2010). In the cytosol and mitochondria, IPP and DMAPP are assembled by the mevalonate (MVA) pathway. Some years ago was discovered an independent pathway in plastids, called the 2-*C*-methyl-*D*-erythritol 4-phosphate (MEP) pathway, nonmevalonate or the 1-deoxy-*D*-xylulose 5-phosphate (DOXP) pathway (EISENREICH et al., 2004). The MEP pathway provides precursors for the synthesis of

isoprene, monoterpenes, diterpenes, carotenoids, abscisic acid, and the side chains of chlorophylls, tocopherols and plastoquinone (SCHUHR et al., 2003).

With the emergence of resistance to so many front line herbicidal compounds, the MEP pathway offers several attractive targets for new molecules discovery efforts. The successful results in studies with clomazone and fosmidomycin, the best-studied inhibitors of the pathway, illustrate the huge benefits that the development of new herbicides based on the MEP pathway might represent (SINGH et al., 2007).

On the basis of this observation, we propose a fast, cheap, reproducible, simple and original *in vivo* assay to identify additional inhibitors of the MEP pathway by measuring the carbon flux through the MEP pathway, having phytoene as an biomarker.

MATERIALS AND METHODS

Barley (*Hordeum vulgare* L.) seeds were sown in moist commercial Metromix potting soil and grown in a dark growth chamber set at 25 °C for four days.

Approximately 0.1g of fresh barley leaves were chopped and incubated in 5 ml of 5 mM MES buffer (pH 6.5) containing 200 μM of norflurazon for 24 h in a growth chamber with the 16/8 light/dark cycle at 25 °C. The various herbicides were tested either at fixed concentration or in different concentrations (DRC – 0,1-100 μM). Phytoene was extracted and quantified according to a protocol based on techniques of Sprecher et al. (1998). The phytoene content was calculated by its extinction coefficient of 1108 mM cm^{-1} and expressed as μg g^{-1} fresh weight.

Phytoene accumulation was plotted against inhibitors concentration to generate dose-response curves. Data were analyzed using a four-parameters log-logistic model using R software. Means and standard deviations were obtained using the raw data and the I_{50} values were obtained from the parameters in the regression curves.

RESULTS AND DISCUSSION

Incubation of barley leaves in medium with norflurazon for 24 h supports the time dependent accumulation of phytoene overtime (Fig. 1). As the accumulation is linear, this assay may be suitable to measure the inhibition of any of the MEP pathway early steps.

The concept of the assay developed in this study is to induce phytoene accumulation, one of the downstream products of the MEP pathway, by inhibition of the enzyme phytoene desaturase with the herbicide norflurazon (a carotenoid biosynthesis inhibitor). The amount of phytoene accumulated is the reflection of the carbon flow through the pathway. Therefore, compounds leading to reduction in phytoene accumulation are potentially inhibiting one of the many enzymatic steps in the early portion of the pathway.

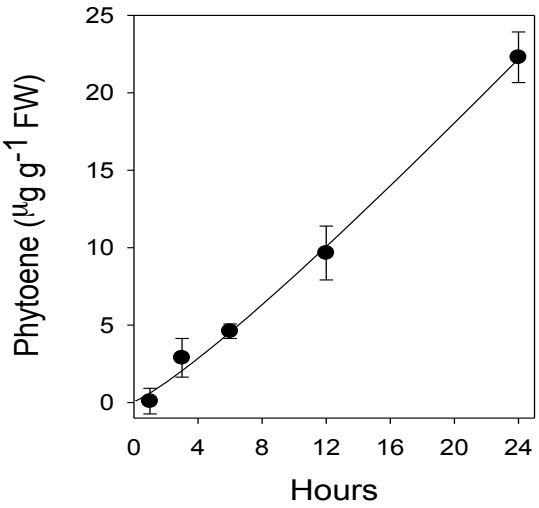

Figure 1. Time-dependent phytoene accumulation in barley exposed to 200 μM norflurazon. Data represent means of three replications with standard deviation.

The effect of clomazone was evaluated and the study in Fig. 2 indicates that the I_{50} is less than 1 μM. For many years clomazone has been utilized as an efficient herbicide and it was later demonstrated that it was not the active molecule but ketoclomazone, a metabolite synthesized *in vivo*, acted as a potent inhibitor (MÜLLER et al., 2000). The requirement for metabolic activation of clomazone was confirmed by repeating the same dose-response curves in the presence of phorate, an organophosphate insecticide that inhibits cytochrome P450 in plants. The addition of 50 μM phorate prevented clomazone from inhibiting phytoene accumulation in greening etiolated barley leaves with I_{50}>100 μM (Fig. 2).

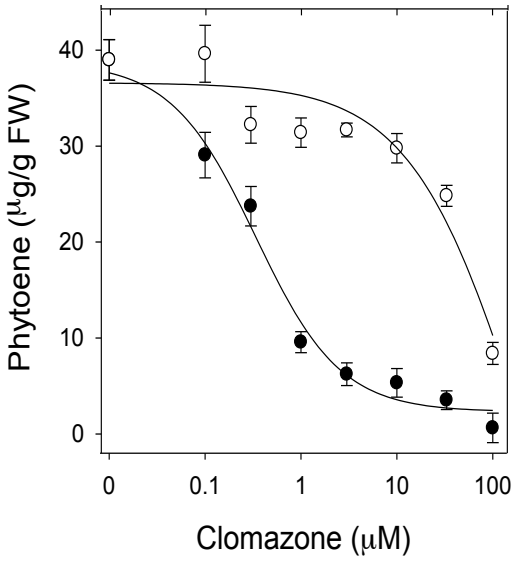

Figure 2. Dose-response curves of clomazone with (○) and without (●) phorate on phytoene accumulation. Data represent means of three replications with

standard deviation.

When tested in the bioassay, surprisingly, the effect of ketoclomazone on phytoene accumulation was less pronounced than observed for clomazone, with I_{50} value of 5 µM (Fig. 3 A). Phorate did not affect the activity of ketoclomazone (Fig. 3 A), this is further confirmation that a clomazone metabolite and not clomazone is the phytotoxic agent and that phorate is preventing its formation. One possible explanation for the high activity of clomazone is that its metabolism generates a still unknown metabolite that is more active than ketoclomazone

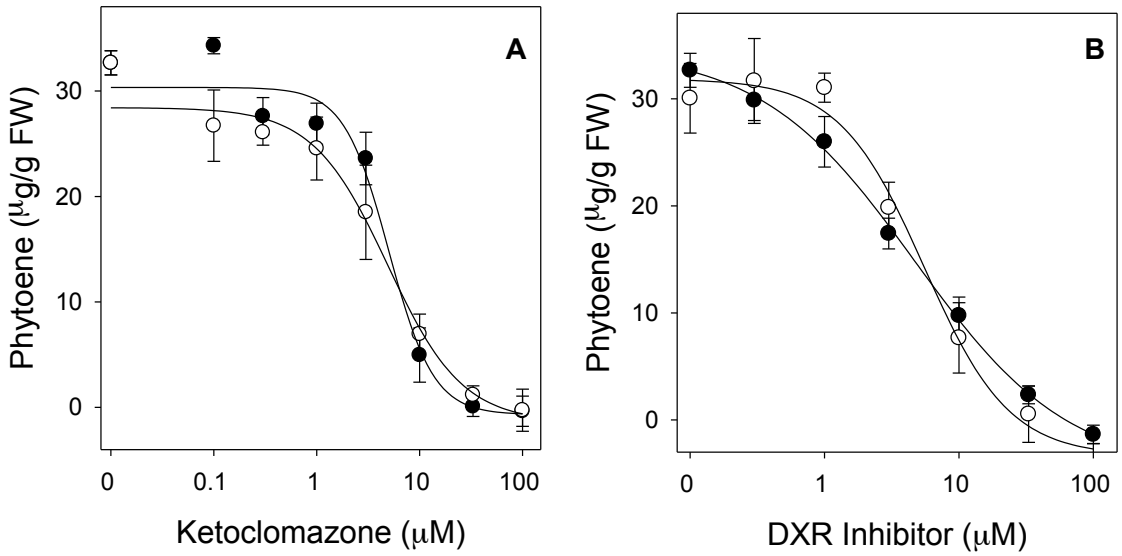

Figure 3. **(A)** Dose-response curves of ketoclomazone with (○) and without (●) phorate, **(B)** Dose-response curves of fosmidomycin (●) and FR-900098 (○) on greening etiolated barley leaves. Phytoene accumulation was induced in the presence of 200 µM norflurazon. Data represent means of three replications with standard deviation.

Since fosmidomycin is known to be a potent herbicide, its effect on the carbon flow toward carotenoids was tested using the described bioassay (Fig. 3 B). As expected, fosmidomycin reduced the formation of phytoene in a dose-dependent manner with an I_{50} value of 5 µM. A considerable number of fosmidomycin derivatives have been synthesized, in order to study the mechanism of its action and identify new promising inhibitors (ERSHOV, 2007). FR-900098, an acetyl derivative from fosmidomycin, significantly blocked carotenoid biosynthesis at approximately 5.5 µM (Fig. 3 B).

CONCLUSION

The bioassay developed in this study proved to be a very useful, simplified and an efficient screening method for MEP pathway inhibitors. The developed method permitted determination of carbon flow through this pathway with accuracy, reproducibility and with minimal samples consumption.

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