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Fluroxypyr—a potential surrogate of 2,4-dichlorophenoxyacetic acid for retarding calyx senescence in postharvest citrus fruit

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ABSTRACT

Searching for an effective and safer alternative to the citrus fruit preservative 2,4-dichlorophenoxyacetic acid (2,4-d) is an issue of common concern in citrus-producing countries. In this study, 27 structural and functional analogues of 2,4-d were preliminarily selected from ZINC database by computer virtual screening. Four varieties of fresh citrus fruits harvested from three consecutive mature seasons were applied in experimental tests, aiming to precisely evaluate the preservation effects of the selected analogues by a comprehensive comparison with the corresponding indices of 2,4-d. The results indicated that fluroxypyr, which has lower toxicity than 2,4-d, significantly reduced calyx senescence and fruit decay without affecting the internal quality of the fruit. Therefore, it can be speculated that fluroxypyr can be an effective and safer alternative to 2,4-d for the preservation of citrus fruits in storage.

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-d), a synthetic plant growth regulator, is commonly used in citrus fruit postharvest industry for its high efficiency to keep fruit quality. However, considering its influence on environment and food safety, its application to citrus fruits is still controversial worldwide. Therefore, searching for safer and more effective new preservatives to substitute 2,4-d is a common issue to be resolved in many citrus-producing countries.

2,4-D has biphasic effects on plant growth. At low concentrations it stimulates cell division and elongation, while at high concentrations it inhibits plant growth and development (Grossmann, 2000; Raghavan et al., 2005). It was used as a phenoxyalkanoic acid herbicide at first. However, since 1950s, it has been widely applied in citrus fruit postharvest handling to maintain fruit quality. As one of the most effective preservatives, 2,4-d can significantly keep citrus fruit button fresh, retard the calyx senescence (Cronjé et al., 2004), reduce fruit weight loss, maintain fruit color and firmness (Sonkar et al., 2009), and prolong storage life. Previous researches have proposed that 2,4-d treatment can remarkably slow down the decline of the contents and activities of antifungal substances in the flavedo, and can significantly inhibit the pathogen infection and decay development (Ben-Yehoshua et al., 1995; Ma et al., 2014) without affecting the internal quality of the stored fruits (El-Otmani and Coggins Jr., 1991).

The application of 2,4-d is restricted in citrus postharvest industry in many countries for its uncertain negative effects on human health and environmental safety, and many main citrus-producing countries have been actively working on developing new alternatives to 2,4-d. Recently, some synthetic auxins such as 3,5,6-trichloro-2-pyridyloxyacetic acid (3,5,6-TPA), 2,4-dichlorophenoxyacetic acid isopropyl ester (Isopropyl 2,4-d ester) and new plant growth regulators such as S-ethyl-4-chloro-*O*-tolyloxythioacetate (MCPA-thioethyl) have been reported to be able to control calyx senescence and reduce fruit weight loss without altering the sensory quality (Carvalho et al., 2008; Sdiri et al., 2013). However,

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very few of them are as effective as 2,4-d and are broadly accepted in the storage of citrus fruits. Hence, it is extremely urgent to search for effective and safer alternatives to 2,4-d.

Virtual screening (VS) is a powerful and inexpensive technology to search potential active compounds from enormous chemical databases with computational methods, which is widely applied in pharmaceutical research (Rester, 2008). VS is divided into ligand-based or structure-based methods depended on the availability of the target structure. When the structure of the protein target is unavailable, ligand-based approaches are often adopted. The ligand-based methods include pharmacophore models, similarity calculations, molecular shapes analysis, and quantitative structure activity relationship (QSAR) models (DDT13-Drawl). Ligand-based pharmacophore modeling is a key computational strategy for facilitating drug discovery in the absence of a macromolecular target structure information (Yang, 2010). Similarity screening is a well-established technique for accessing chemical structure databases, utilizing the known actives as input information to identify other structurally diverse compounds with similar properties (Hert et al., 2006). Usually, experimental test (wet lab) needs to be carried out to confirm the results of VS hit list (dry lab). Despite that virtual screening is broadly utilized in drug development, there are hardly any reports of its application in the development of food preservatives so far.

In this research, the structural analogues of 2,4-d were further screened from an enriched subset of compounds which were firstly filtered from the databases of SPECS, Maybridge and ChemDiv by similarity searching and pharmacophore matching from Zinc. In addition, functional analogues selected from commercial auxin-like herbicides with similar functions to herbicidal 2,4-d were purchased from market and used as a kind of candidates. The preservation effects of these two kinds of analogues were experimentally tested finally with different varieties of citrus fruits, and the predictive toxicity of them was used as an important index to further identify the alternatives to 2,4-d.

2. Materials and methods

2.1. Pharmacophore matching and similarity searching for structural analogues of 2,4-d

The pharmacophore matching and substructure-similar compounds were screened from a free compound database ZINC (http://zinc.docking.org) (Irwin and Shoichet, 2005) according to the previously reported methods (Gao et al., 2010; Pirhadi et al., 2013). In pharmacophore virtual screening process, phenol, chlorobase and carboxyl were set as the searching pharmacophore feature, and only the most similar compounds were selected for further experiments. Similarity screening process was divided into several steps. To enrich the molecules with physically close property and reduce the library size, compounds with a molecular weight = [200,300], an ALogP = [2,3], a molecular solubility with LogS larger than -4 were preliminarily calculated and filtered out using Pipeline Pilot 8.5 (Accelrys Software Inc.-San Diego, CA). Then, the enriched subset was analyzed with Surflex-Sim of SYBYL program package (SYBYL-X 2.0, Tripos Inc., St. Louis, MO, USA) to calculate their similarities with 2,4-d. Only the compounds with scores higher than 7 were selected according to a previous report (Jain, 2004). Finally, the toxicity of the hit compounds was evaluated with Toxicity Prediction by Komputer Assisted Technology (TOPKAT; Accelrys Inc., Birmingham, MA).

2.2. Reagents

The information of all chemicals used in this research including names, CAS numbers, origins, manufacturers, purities, structural formulas, and the similarities to 2,4-d was shown in detail in supplemental Table 1. 2,4-D and its structural and functional analogues were dissolved into water at the effective concentration of 0.2 mmol L^{-1}.

2.3. Sampling, treatment and storage of fruits

Fruits of four citrus varieties were selected to test the preservation effects of these candidates, including Satsuma mandarin (*Citrus unshiu* Marc.), Lane Late navel orange (*Citrus sinensis* (L.) Osbeck), Olinda Valencia orange (*Citrus sinensis* (L.) Osbeck), and Newhall navel orange (*Citrus sinensis* (L.) Osbeck). Commercially mature fruits were harvested from the orchard of Huazhong Agricultural University in Wuhan, Hubei Provence, China, in the years of 2012–2014. Fruits uniform in color and size and free from any visible injury or blemishes were collected for next experiments.

Sample fruits were equally divided into several groups and each group (about 100 kg) was dipped in the solution with an effective concentration of 0.2 mmol L^{-1} for 2 min, then stored in a ventilated warehouse. Water-treated fruits were set as negative control (CK), and 2,4-d treated fruits were set as positive control (2,4-d).

2.4. Evaluation of the rates of fruit decay and calyx senescence

Fruit decay and calyx senescence were evaluated visually. Respectively 80 healthy fruits from the CK and each analogue-treated group were randomly selected and equally divided into two groups (40 fruits for each biological replication). Fruit decay incidence was expressed as the average percentage of rotted fruit. And calyx senescence rates were calculated according to a reported method (Carvalho et al., 2008), and displayed as the average of the two replicates.

2.5. Measurement of fruit weight loss

The rate of fruit weight loss was measured according to the previous report (Chaudhary et al., 2012) with some modification. Twenty fruits were randomly selected and marked from each group. Weight of each fruit was regularly recorded from 0 day after treatment by using electronic analytic balance ($\pm 1.0 \times 10^{-5}$ kg accuracy, JA5003, Shanghai Balance Instrument Plant, Shanghai, China). Fruit weight loss rate was displayed as the average weight loss rate of these 20 replicates.

2.6. Measurement of fruit firmness

Fruit firmness was determined according to a previously reported method (Sdiri et al., 2012) using a penetrometer (FT02, Italy) fitted with a 1.5 mm diameter probe tip. Each fruit was measured 6 times on 6 equatorial fruit zones at intervals of about 60°. The results were shown as the average firmness of 20 fruits and expressed in Newton (N).

2.7. Measurement of fruit internal quality

For each treatment, a total of 30 fruits were collected for three biological replications. Juice squeezed from each 10 fruits was filtered through four layers of gauze. The total titrable acidity (TA) in juice was detected by fruit acidity meter (model: GMK-835F orange) according to the instructions. Total soluble solid (TSS) in juice was measured by digital hand-held pocket refractometer (PAL-1; Atago, Japan) and three replicates were performed.