Industrial Crops and Products 81 (2016) 202–210

Contents lists available at ScienceDirect

Industrial Crops and Products

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

Thidiazuron increases fruit number in the biofuel plant *Jatropha curcas* by promoting pistil development

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ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form
17 November 2015
Accepted 20 November 2015
Available online 10 December 2015

Keywords:
Biofuel
Cytokinin
Flower sex
Pistil development
Stamen abortion
Thidiazuron

ABSTRACT

Jatropha curcas L. is a potential biofuel plant because the composition of its seed oil is suitable for biodiesel and bio-jet fuel production and it is able to grow in unproductive subtropical or subdesert soils. Many studies have been performed to improve the seed yield of *J. curcas* to meet the needs of the biodiesel industry. As female flower number is an important factor affecting seed yield, an increase in the number of female flowers through the modification of sex expression is critical to the improvement of *J. curcas* for use as a biofuel. In this study, thidiazuron (TDZ), a synthetic compound with cytokinin (CK) activity, was exogenously applied to inflorescence meristems in four developmental stages to study its effect on sex expression in *J. curcas*. The results revealed that TDZ treatments of 75 μM and 225 μM promoted pistil development, which significantly increased the number of female flowers along with the development of inflorescence meristems. Number of female flowers reached a peak (40.0 female flowers per inflorescence) at 225 μM TDZ on stages III and IV inflorescence meristems. TDZ also reversed stamen abortion in stages II, III, and IV female flowers and induced bisexual flowers, which largely depends on the development stage of the inflorescence meristems. Furthermore, TDZ treatment increased the branch orders of the dichasia on the inflorescence, as observed by scanning electron microscopy. However, the total number of flowers was significantly decreased, as a result of the abortion of flower buds caused by TDZ. The number of mature fruits, which determines seed yield, was significantly increased by TDZ treatment, although this treatment resulted in a greater number of premature fruits. This study found that treatment with TDZ improved the fruit number of *J. curcas* by promoting pistil development. TDZ may play dual roles in the determination of flower sex, i.e., promoting pistil development and reversing stamen abortion in female flowers, which could shed light on the mechanism of sex determination in *J. curcas* and/or other non-model plants.

1. Introduction

In the context of increased demand for biofuels, the potential of the non-food species *Jatropha curcas* L. (hereafter referred to as *Jatropha*) has been recognized because the quality of its seed oil is suitable for biodiesel production and it is able to grow in unproductive subtropical or subdesert soils (Edrisi et al., 2015; Sujatha et al., 2013). Inflorescences of *Jatropha* are composed of five to nine dichasia on the upper part of the inflorescence rachis and one or two secondary inflorescences at the base of the inflorescence rachis (which also include 1–3 dichasia) (Supporting information Fig. S1a) (Fresnedo-Ramírez, 2013). There are four orders of branches on each dichasium, and the sex of the flower is dependent on its location on the four orders of the branches. The terminal flower produced at the joint of the first order of dichotomous branching is usually female (designated as the 1st flower, Supporting information Fig. S1b); flowers produced at the joint of the second dichotomous branching (the 2nd flowers, Supporting information Fig. S1b) may be female or male, which varies from plant to plant and population to population in response to time, climate, and nutrition; all flowers produced at the joint of the third and fourth dichotomous branching are male (the 3rd and 4th flowers, respectively; Supporting information Fig. S1b) (Wu et al., 2011). Therefore, female flowers are very limited in *Jatropha*, and increasing the number of female flowers through the modification of sex expression seems critical for the improvement of seed yield (Nietsche et al., 2015).

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http://dx.doi.org/10.1016/j.indcrop.2015.11.054
0926-6690/

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2015.11.054.

Plants have evolved various mechanisms to promote cross-pollination, including the production of unisexual flowers on the same or different plants. Approximately 10% of angiosperms produce unisexual flowers (Yampolsky, 1922), and two broad categories of unisexual flowers have historically been recognized. In one type (type I), flowers become unisexual through the abortion of male or female reproductive organs, and in the second type (type II), flowers are unisexual from inception, as sex differentiation occurs before the initiation of stamens and carpels (Mitchell and Diggle, 2005). Most *Jatropha* plants produce unisexual flowers in inflorescences (Carels, 2009; Fresnedo-Ramírez, 2013), and Wu et al. (2011) reported that female and male flowers are formed via different mechanisms in *Jatropha*. The female flower is a type I unisexual flower that exhibits bisexual organs upon initiation, in which there is a rudiment of the nonfunctional male organ, whereas the male flower is a type II unisexual flower that bears no vestigial sexual organ during development. The existence of these two modes is also supported by the two whorls with five minute staminodes each at the base of the ovary, which develop into functional stamens, causing the *Jatropha* flower to sometimes become bisexual (Nair and Abraham, 1962).

Unisexual flower formation has been reported to be regulated by various phytohormones (Gerashchenkov and Rozhnova, 2013; Zhang et al., 2014), among which cytokinin (CK) has been shown to have a feminizing effect on a number of plant species. For example, the exogenous application of CK converted male flowers to hermaphroditic flowers in *Vitis vinifera* (Negi and Olmo, 1966) and induced female flowers in *Momordica charantia* (Ghosh and Basu, 1982), *Luffa acutangula* (Bose and Nitsch, 1970) and *Luffa cylindrica* (Takahashi et al., 1980). We have previously reported that 6-benzyladenine (BA, a synthetic compound with CK activity) treatment significantly increased the number of female flowers per inflorescence and induced bisexual flowers (Pan and Xu, 2011), which may have resulted from the differential expression of a large number of genes, such as those related to phytohormone biosynthesis and signaling and the regulation of the cell cycle (Chen et al., 2014; Pan et al., 2014). However, BA treatment produced too many flowers (both male and female), most of which were not well developed, and did not contribute to final seed yield. Thidiazuron (TDZ), a diphenylurea derivative, has been reported to have a high degree of intrinsic CK-like activity, much higher than that of BA (Thomas and Katterman, 1986), and it functions as an inhibitor of CK oxidase activity mainly through a non-competitive mechanism that is different from that of BA (Kieber and Schaller, 2014). Here, we report the effects of TDZ on sex expression in *Jatropha* through the exogenous application of various concentrations of TDZ onto inflorescence meristems (IMs) at four developmental stages. Our results indicate that TDZ may play dual roles in flower sex determination, i.e., promoting pistil development and reversing stamen abortion in female flowers, resulting in an increase in the number of fertile flowers (female and bisexual flowers) and, consequently, fruits. This study may shed light on the mechanism of sex determination in *Jatropha* and/or other non-model plants.

2. Materials and methods

2.1. Plant materials and growth conditions

Jatropha cuttings from a local population were grown at the beginning of March 2011 in a field at the Xishuangbanna Tropical Botanical Garden (XTBG; 21°54′N, 101°46′E; 580 m in altitude) of the Chinese Academy of Sciences located in Mengla County, Yunnan Province, southwestern China. The average rainfall, temperature and relative humidity in April 2012 and 2013 when the experiments were conducted were 41.3 mm, 23.9 °C and 75%, and 142.9 mm, 23.7 °C and 80%, respectively (data from Xishuangbanna Station for Tropical Rain Forest Ecosystem Studies). Plants were monocultured at a density of 1.5 m × 3 m under normal fertilization.

2.2. Thidiazuron (TDZ) application

A stock solution (454.0 mM) of thidiazuron (TDZ, Bio Basic Inc., Toronto, Ontario, Canada) was prepared by dissolving 0.1 g of TDZ with 1 mM NaOH and bringing the final volume to 1 ml with distilled water. Tween-20 (Polysorbate-20, Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China) was added at a final concentration of 0.05% (V/V) as a wetting agent to all of the TDZ working solutions. Inflorescence meristems (IMs) in four developmental stages (Supporting information Fig. S2) were used for TDZ application, as the stages may respond differently to TDZ treatment: stage I—the 1st flower appeared the primordial stamen (Supporting information Fig. S3a); stage II—the 2nd flower appeared the primordial stamen (Supporting information Fig. S3b); stage III—the 3rd flower appeared the primordial stamen (Supporting information Fig. S3c); stage IV—the 4th flower appeared the primordial stamen (Supporting information Fig. S3d). Three milliliters of each of the various concentrations of TDZ working solutions (0, 25, 75 and 225 μM) that contained equal volumes of 1 mM NaOH and 0.05% (V/V) Tween-20 were sprayed on each IM and its surrounding leaves using a hand sprayer. Spraying was conducted once on a sunny day. Thirty-five IMs were used for each treatment. The results of this study were confirmed by two replicated experiments at the end of April 2012 and 2013.

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2015.11.054.

The total number of flowers and number of each sex type per inflorescence and the number of fruits per infructescence were counted. A female flower was defined as a flower with only a pistil, and a male flower was defined as a flower with only stamens. Bisexual flowers were defined as flowers with both pistils and visible stamens, and an aborted flower bud was defined as a flower bud observed in the early inflorescence development stage after TDZ treatment that was unable to bloom and eventually withered. The total flower number is the sum of the male, female, and bisexual flowers. A mature fruit contained mature seeds, whereas an immature fruit contained small seeds that were unable to mature.

2.3. Scanning electron microscopy (SEM)

Stages I and IV IMs were collected 6 days after TDZ treatment and fixed overnight in FAA (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) and dehydrated through a graded ethanol series to 100%. Materials were critical point dried using liquid CO_2, mounted on aluminum stubs with double-sided tape, gold-coated with an Edwards S150B sputter coater, and then examined through a scanning electron microscope (EVO LS10, Germany, at 10 kV).

2.4. Characterization of seeds

After being air-dried for 2 months, seeds from control and TDZ-treated plants were analyzed to determine their weight and oil content. Seed oil contents were determined with a minispec mq-one Seed Analyzer (Bruker Optik GmbH, Germany) with *Jatropha* seed oil used as a reference.

2.5. Statistical analysis

Data were analyzed using Statistical Product and Service Solutions software (SPSS Inc., Chicago, IL, USA, version 16.0). Differences