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Optimization of D-pinitol extraction from vegetable soybean leaves and its potential application in control of cucumber powdery mildew

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ABSTRACT

D-pinitol is a bioactive compound with an important application in both food and nonfood industries. In the present study, the leaves of twelve vegetable soybean cultivars that are usually treated as agricultural waste were screened for the presence of D-pinitol. The results showed that the D-pinitol content in these 12 cultivars ranged from 1.32 to 3.04% (w/w) of dry weight, and the cultivar Z98002 was found to contain the highest amount of D-pinitol. Response surface methodology (RSM) was used for optimization of D-pinitol extraction conditions by the Box–Behnken design. The predicted extraction efficiency, which is at least 1.3-fold that of the previously reported typical method, reached to 3.27% under the following optimal conditions: temperature of 65.5 °C, extraction time of 86.8 min, and dilution rate of 1:10. The test in the growth chamber showed that the efficacy of soybean extracts in cucumber powdery mildew control was dose-dependent on the D-pinitol concentration, and the formulated extracts clearly increased the control compared to the crude extract. This effect of the D-pinitol formulation in a greenhouse was confirmed in four provinces of China over a period of two years. Our present study provides the optimized extraction conditions for a selected agricultural waste—vegetable soybean—to obtain a high yield of D-pinitol. Results from the growth chamber test and the greenhouse bioassay will be useful in exploring a low-cost phytochemical fungicide for cucumber powdery mildew control.

1. Introduction

D-pinitol is a bioactive compound reported to have important biological and medical activities. Many studies have shown the beneficial effects of D-pinitol on human metabolism because of its insulin-like function (Kim et al., 2007). The compound also has some biocontrol effect on butterfly, mosquito, and moth by oviposition attraction, larvicidal activities, and growth inhibition (Dreyer et al., 1979; Chaubal et al., 2005; Honda et al., 2012). Another important physiological function of D-pinitol is as an osmotic tolerance molecule associated with drought and salinity stresses in soybean (Silvente et al., 2012). The accumulation of D-pinitol in stressed plants is believed to be beneficial for stress adaptation, membrane stabilization, and osmotic alteration (Popp and Smirnoff, 1995). This substance was first isolated from *Pinus lambertiana* (Gottlieb and Brauns, 1951); it was later found to be abundant in leaves of *Mesembryanthemum*, Leguminosae, and Caryophyllaceae and to be present in low levels in other plant families (Shaik et al., 2011). In soybean leaves, it is a major low molecular weight carbohydrate combined with other cyclitols of smaller amounts (Phillips and Smith, 1974; Phillips et al., 1982).

The soybean *Glycine max* is one of the most important grain legumes worldwide, with 200 million metric tons produced per year. It provides excellent vegetable proteins, abundant plant oil, and other essential nutriments, such as isoflavones and phenolic compounds (Tyug et al., 2010; Silvente et al., 2012). Soybean is used not only to produce various food products, for instance, soy milk, soy flour, tofu, vegetable pods, soybean oil, and so on, but also to form root nodule symbiosis with nitrogen-fixing bradyrhizobia to fertilize agricultural land (Silvente et al., 2012). The vegetable soybean *G. max* (L.) Merr is a specialty soybean harvested as a fresh vegetable before green pods turn yellow at the R6 stage (before seed maturity) of soybean development. The seeds of vegetable soybeans are larger, sweeter, and more tender compared to those of grain soybeans (Zhang et al., 2010). In Asia, especially in Japan, China, Korea, and Southeast Asia, vegetable soybean is a very popular vegetable and snack food because it is rich in minerals and vitamins A and B, with a slightly sweet taste and mild flavor (Zhang

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et al., 2010; Chen et al., 2011). Japan has the largest consumption of edamame (the Japanese name of vegetable soybean) in the world at 160 thousand tons per year. The United States imports more than 10 thousand tons of frozen soybeans each year. Vegetable soybeans have also been cultivated in the United States for many years because of their high profit and broad market (Lin, 2001; Rao et al., 2002; Ma et al., 2004). In recent years, considerable research has been reported on vegetable soybeans, particularly with respect to edibility, nutrition, trait transmission, and storage processing (Rao et al., 2002).

China has the earliest history of vegetable soybean development, dating back to more than one thousand years ago (Gai and Guo, 2001). Vegetable soybean has been a very important agricultural product in areas in the middle and downstream of the Yangtze River and in the southern and eastern littoral of China, where the main areas of vegetable soybean production, consumption, and export are also found. Traditionally, the pods with beans are harvested for eating, whereas the rest, including the fresh leaves and stems, are discarded as agricultural waste. Considering the high content of D-pinitol in soybean leaves, in this study, the leaves of twelve vegetable soybean cultivars originated from major production areas in China were analyzed for D-pinitol content by gas chromatography (GC), and the optimal extraction conditions were established by response surface methodology (RSM).

RSM is a collection of experimental strategies, and mathematical and statistical methods to optimize the independent factors (response variable) via less number of experiments (Ahmad et al., 2012; Wang et al., 2013). The visual representation of variable interactions was shown by 3D contour plots. This method has been applied widely in the extraction of bioactive components from natural materials (Li et al., 2010; Turhan, 2011; Wang et al., 2013). Box–Behnken design is rotatable second-order designs based on three-level incomplete factorial designs. Box–Behnken design requires an experiment number according to $N = k^2 + k + c_p$, where (k) is the factor number and (c_p) is the replicate number of the central point (Aslan and Cebeci, 2007). In this study, the Box–Behnken experimental design was chosen to optimize the extraction conditions by three independent variables: extraction temperature, extraction time and dilution rate.

Based on the control effect of D-pinitol on cucumber powdery mildew disease (Wang et al., 2012), the efficacy of the soybean extract and its formulation in the control of this plant disease was evaluated in a growth chamber, and the D-pinitol formulation was evaluated in greenhouses in four provinces of China over a period of two years. This work aimed to obtain a high yield of D-pinitol and to explore a potential botanical fungicide recycled from agricultural waste.

2. Materials and methods

2.1. Plant material

Plant genotypes were selected from and bred in the middle and lower Yangtze River and the southern and eastern littoral of China. The following twelve cultivars were tested in this study and the identification was omitted: JD095, JD083, JD595, Z98002, ZH0526, Z5, KF761, LL9, X3, QS2, KKY12, and L04035. The seeds of the twelve cultivars were sown on the same farm in a suburb of Shanghai on 16 April 2009 and were grown under the same conditions. The plant tissue of leaves, stems, and pods were collected in July, 88 days after planting, and the fresh beans were harvested. The collected sample was dried at 40 °C immediately after collection and then ground. The powder samples were passed through a 40-mesh sieve to obtain 0.35 mm particle size and kept for further experiments.

Table 1
Three level Box–Behnken experimental design for D-pinitol extraction from vegetable soybean and results.

Test no[a]	Temperature (°C)	Time (min)	Dilution rate (w/V)	Contents (%)
1	50	80	1:30	2.65
2	90	80	1:30	2.77
3	70	80	1:20	2.89
4	70	80	1:20	2.89
5	50	120	1:20	2.79
6	90	80	1:10	3.08
7	90	120	1:20	2.59
8	70	80	1:20	2.97
9	70	40	1:10	3.11
10	70	80	1:20	3.02
11	70	120	1:30	2.81
12	70	80	1:20	2.93
13	50	80	1:10	3.12
14	70	120	1:10	3.19
15	90	40	1:20	2.59
16	50	40	1:20	2.72
17	70	40	1:30	2.75

[a] The order of test was generated using Design Expert (version 8.0.6; Stat-Ease Inc., Minneapolis, USA).

2.2. Standard solutions of D-pinitol and content test of 12 Chinese soybean cultivars

Standards of D-pinitol were purchased from Sigma Co. (Sigma, China) and diluted in distilled water to obtain working solutions at concentrations of 0.2, 0.6, 1, 1.5, and 2 mg ml^{-1}. Each D-pinitol working solution (0.1 ml) was passed through a 0.2-mm nylon membrane filter and dried under vacuum. Analysis was carried out by gas chromatography (GC) after derivatization with trimethylsilylimidazole (TMSI) (Sun et al., 1999). The column temperature was maintained at 150 °C for 3 min, increased to 200 °C at 5 °C/min, and then further raised to 300 °C at 10 °C/min. The final temperature of 300 °C was maintained for 17 min. The carrier gas was purified helium at 30 cm/s. The curve of the peak area (Y) vs. the D-pinitol standard concentration (X) was plotted. The linear regression equation was: $Y = 747{,}744X + 772$, $R^2 = 0.9994$. For each cultivar, a 4-g sample of powdered leaves was extracted at 70 °C for 1 h in water at a ratio of 1:20 (w/v, powder weight/solvent volume). The extract was filtered through Whatman no. 1 filter paper, and the filtrate was adjusted to 100 ml with distilled water. Samples of 0.1 ml each from the twelve cultivar extracts were tested, and the objective peak areas were recorded. The D-pinitol concentrations corresponding to the peak areas were calculated with the standard curves obtained above. The experiments were repeated three times, and the average concentrations were recorded. The D-pinitol content of each cultivar was converted by the following formula:-

$$\text{Content percentage } (\%) = (\text{average concentration} \times \text{volume} \times 100)/\text{powder weight},$$

where volume = 100 ml, and powder weight = 4 g.

The cultivar found to have the highest D-pinitol content was selected for comparison of different content levels among tissues from leaves, stems, pods, and beans. Finally, the plant tissues of the