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Note

Comparative metabolome analysis of seed kernels in phorbol ester-containing and phorbol ester-free accessions of *Jatropha curcas* L.

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Abstract *Jatropha curcas* L. (Jatropha) is a promising source of renewable bioenergy, since its abundant seed oil can readily be converted into biodiesel. However, industrial use of Jatropha seed oil is problematic; the toxicity of the seeds and oil endangers the oil users and prevents the use of byproducts, such as seed cakes. The most toxic compounds in Jatropha are phorbol esters (PEs), tetracyclic diterpenoids known for their tumor-promoting activity. It is important to utilize a non-toxic accession of Jatropha that lacks PEs in industrial feedstocks. Here, we used an LC-Orbitrap-MS system to characterize the metabolome of seed kernels from PE-containing and PE-free accessions of Jatropha. Among the more than 12,000 metabolites detected, 18 were specific to the PE-containing accession, and most of these appeared to be PEs or derivatives thereof. In contrast, only four ions were specific to the PE-free accession. These results indicate that PE-containing and PE-free Jatropha are broadly similar in their metabolism, but that the PE-containing accessions undergo PE biosynthesis.

Key words: *Jatropha curcas* L., metabolome analysis, non-toxic accession, phorbol ester, seed kernel.

Due to concerns about the environment, the worldwide demand for biofuels, such as bioethanol and biodiesel, is increasing. Since first generation biofuels are mainly produced from crop-derived starch, sugar, and oil, biofuel production competes with food cultivation and contributes to environmental damage. To build a sustainable society, we have to overcome these problems by developing new types of biofuels, such as second generation biofuels (made from non-food crops) and third generation biofuels (made from algae) (Singh et al. 2011).

In this respect, *Jatropha curcas* L. (Jatropha) is a promising feedstock material for biodiesel production. Jatropha is a member of the Euphorbiaceae family, which is distributed in the tropics and subtropics, and is considered to be a multipurpose plant that is valuable not only for its use as an oilseed crop, but also for its medicinal properties. Since the biodiesel made from Jatropha oil has properties similar to conventional petrodiesel, it can be used in existing diesel engines (El Diwani et al. 2009; Raja et al. 2011; Singh and Padhi 2009). This fast growing shrub, which is readily propagated and exhibits stable productivity, starts producing fruit from the second year. These features make Jatropha an attractive non-food crop for biofuel production (Heller 1996). The only drawback of this crop is the toxicity of its seeds and/or oil, which present safety concerns for the use of the oil and its byproducts, e.g., seed cakes. The Jatropha seed contains several different toxic substances, including a lectin (curcin), protease inhibitors, saponins, and phorbol esters (PEs) (Makkar et al. 1998; Martínez-Herrera et al. 2006). Among these compounds, the PEs are most problematic. PEs, which are known for their tumor-promoting activity, are polycyclic compounds in which two hydroxyl groups bonded to neighboring carbon atoms are esterified to fatty acids (Goel et al. 2007). Six types of PEs, Jatropha factors C1–C6, have been determined as diterpene esters, which possess the common diterpene 12-deoxy-16-hydroxyphorbol (Adolf et al. 1984; Haas et al. 2002; Supplemental Data 1). Makkar et al. (1998) and Makkar and Becker (1999) demonstrated that the toxicity of Jatropha seeds is largely attributed to the presence of PEs, and not to lectin, as previously suggested (Stirpe et al. 1976). Thus, to obtain maximum benefits from Jatropha oil, it is important to use PE-free accessions of Jatropha that lack PE as the feedstock for biodiesel production. However, how different PE-containing and PE-free

Abbreviations: Jatropha, *Jatropha curcas* L.; LC-MS, liquid chromatography mass spectrometry; PDA, photodiode array; PE, phorbol ester.
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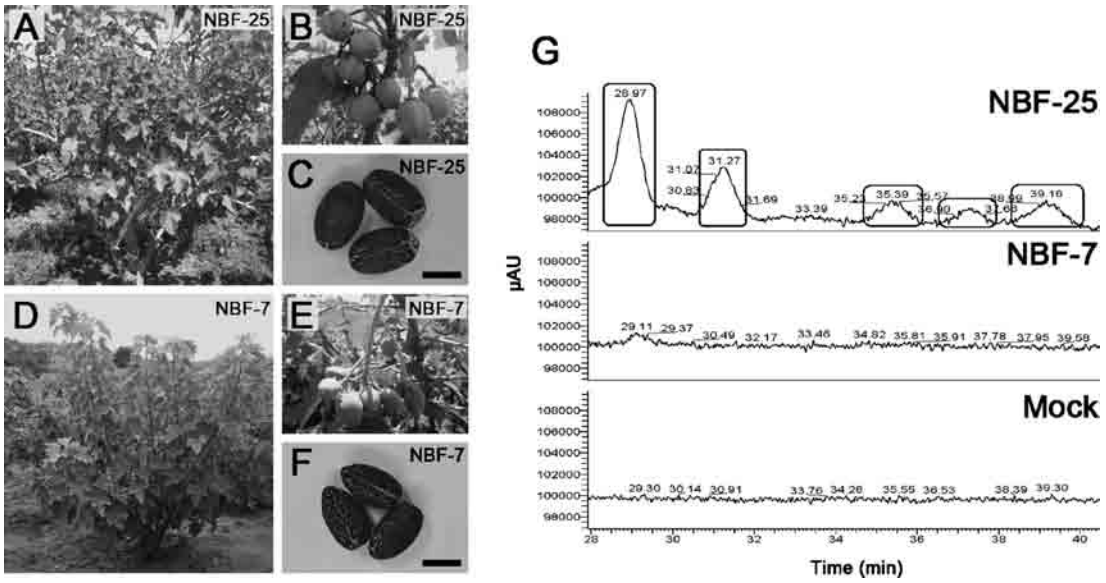

Figure 1. Characterization of PE-containing (NBF-25) and PE-free (NBF-7) accessions of Jatropha. Tree forms (A, D), fruit clusters (B, E), and seeds (C, F) of NBF-25 (A–C) and NBF-7 (D–F) are shown. Bars = 1 cm (C, F). (G) Chromatogram of seed kernel extracts on PDA of selective wavelength (279.5 to 280.5 nm) obtained from NBF-25 (upper), NBF-7 (middle), and mock (bottom) samples.

accessions are in broad metabolic aspects is still unclear.

To clarify the metabolic characteristics of PE-free Jatropha seeds, we performed a comprehensive metabolome analysis of seed kernels of PE-containing (NBF-25) and PE-free (NBF-7) accessions of Jatropha. Both of these accessions of Jatropha were collected by Nippon Biodiesel Fuel Co., Ltd. (http://www.nbf-web.com/index.html) from Latin America, and selected by the PE amounts in the seed oil. These accessions have similar tree forms, but NBF-7 undergoes less branching in the first year following planting (Figures 1A, D). For the metabolome analysis, seeds of these accessions (Figures 1C, F) were subjected to LC-Orbitrap-MS analysis. Seed coats were removed and the kernels were frozen in liquid nitrogen. Subsequently, the kernels were ground to a fine powder using a mortar and pestle. Approximately 150 mg powder sample was dissolved in three volumes of 100% methanol, and the solution was shaken (25 Hz, 2 min) using a TissueLyser (Qiagen) to extract seed kernel metabolites. After the extraction, the sample was centrifuged at 4°C for 10 min at 15,000 rpm. The supernatant was collected and filtered through a 0.2-μm Millex LG filter (Millipore), to yield the final sample. The assay was performed using a Finnigan LTQ ORBITRAP XL spectrometer (Thermo Scientific) connected to an Agilent 1200 series liquid chromatography system (Agilent). To determine the quantity of PE in seed kernels, phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) was added to the final seed kernel extracts as the standard. The experimental conditions are presented in Supplemental Data 2.

Figure 1G shows typical chromatograms of seed kernels on photodiode array (PDA) with selective wavelength (279.5 to 280.5 nm). Extracts of the NBF-25 seed kernels showed five peaks, which are highlighted with black boxes (upper panel of Figure 1G). Subsequent mass spectrometry indicated that these five peaks are derived from the $[M+H]^+$ fragments at m/z 711, which correspond to PEs (Supplemental Data 3). The peaks resulted in the accurate mass of m/z 711.3893, which gave the empirical formula of C44H54O8 at 0.09 ppm error (peak16 in Table). Thus, the five peaks on the PDA chromatogram of NBF-25 originated from PE. Based on the PDA chromatograms, the total PE content of the NBF-25 seed kernels was determined to be 1.53 ± 0.15 g/kg (average ± SD; in PMA equivalent). In contrast, there was no peak on the PDA chromatogram (middle panel of Figure 1G) or on the mass chromatogram (Supplemental Data 3) in the NBF-7 extract. These data demonstrate that NBF-7 seed kernels do not contain a detectable amount of PE. Given the background signal level, the PE amount in NBF-7 was estimated to be lower than 0.03 g/kg in PMA equivalent.

Next, the metabolomic profiles of NBF-25 and NBF-7 were compared. From data of 12,739 compounds with retention times of 8 to 80 min, we selected 2,592 compounds that were reproducibly detected for further analysis. A statistical analysis revealed that 60 metabolites showed significant quantitative differences between NBF-25 and NBF-7 (Student's t-test; $p<0.01$). Combined with data from the detailed mass chromatogram analysis, 22 ions were finally identified as accession-specific metabolites (Table 1; Supplemental Data 4). Eighteen of these ions (No. 1–18) were NBF-25-specific, including Jatropha PEs (Jatropha factor C1–C6; peak 16), and the other four ions (No. 19–22) were specific

Table 1. Accession-specific ions in seed kernels.

No.	*m/z*	Possible Molecular Formula	Comments
NBF-25 (PE-containing Jatropha) specific ions			
1	311.1643	C20H22O3	In-source fragment of PE
2	317.2112	C20H28O3	Fragment ion of No. 3
3	319.2269	C20H30O3	
4	383.2217	C24H30O4	In-source fragment of PE
5	501.2272	C31H32O6	Fragment ion of No. 6
6	519.2377	C31H34O7	
7	529.2949	C34H40O5	In-source fragment of PE
8	545.2534	C33H36O7	
9	631.3782	C43H50O4	In-source fragment of PE
10	657.368	C44H48O5	In-source fragment of PE
11	665.3838	C43H52O6	
12	675.368	C44H50O6	In-source fragment of PE
13	691.363	C44H50O7	Fragment ion of No. 15
14	693.3786	C44H52O7	In-source fragment of PE
15	709.3736	C44H52O8	
16	711.3893	C44H54O8	PE (Jatpropha factors C1–C6)
17	735.4256	C47H58O7	
18	956.631	C51H89NO5	
NBF-7 (PE-free Jatropha) specific ions			
19	310.3105	C20H39NO	Fragment ion of No. 20
20	328.3211	C20H41NO	
21	507.2278	C32H30N2O4	
22	662.5719	C41H75N1O5	

to the PE-free accession, NBF-7. Retention times (28.9–45.24 min) of these ions suggest that 10 ions specific to NBF-25, i.e., No. 1, 4, 6, 7, 9, 10, 12, 14, 16, and 17, are related to PE (Supplemental Data 4). The accurate mass data and MS/MS patterns suggested that No. 12 and 14 are in-source fragment ions of PE ($-2H_2O$ and $-H_2O$ ions, respectively). The MS/MS analysis of these peaks, revealed that No. 1, 4, 7, 9, and 10 would be also derivative ions by in-source fragmentation of PE (Table 1). In addition, the presumed molecular formula suggests that No. 15 has a molecular structure with one more degree of unsaturation than PE (Table 1). Mass chromatograms of No. 15 (and its putative fragment ion, No. 13) contained numerous peaks of isomeric forms that had different retention times from PE (Supplemental Data 4). Thus, No. 13 and 15 are thought to be derived from substances distinct from the PEs already reported, but they might be related to PE. Unfortunately, we could not identify the other compounds. However, an *in silico* metabolite database search using the molecular formula of No. 19–22 showed that the NBF-7-specific ions are different from all known toxic substances (KNApSAcK; http://kanaya.naist.jp/KNApSAcK/; Ohta et al. 2007).

The fact that few accession-specific metabolites were identified indicates that there is little metabolic difference between NBF-25 and NBF-7, except with respect to PE biosynthesis. A similar conclusion was reached in previous works on different accessions of Jatropha, in which no significant qualitative difference was found between toxic and non-toxic Jatropha, other than PE content (Makker and Becker 1997; Makker et al. 1998; Martínez-Herrera et al. 2006). Since PEs are strong irritants of the skin (Adolf et al. 1984; Hirota et al. 1988), seed oil from PE-free Jatropha should be much safer for oil users than that from PE-containing accessions. Moreover, it has been reported that Jatropha seed kernels are rich in protein; specifically, the level of essential amino acids, except lysine, is higher than in the FAO/WHO reference protein. Therefore, the seed cake formed after oil extraction may potentially be used as a protein-rich ingredient in livestock feeds or for human consumption (Makker et al. 1998; Martínez-Herrera et al. 2006). Previous feeding tests revealed that PE and lectin are two main factors that inhibit the digestion of Jatropha meals. Whereas relatively mild heat treatment (121°C, 66% moisture, 15 min) deactivates lectin to negligible levels, alkali-heat treatment and/or alcohol extraction are required for the removal of PE (Makkar and Becker 1997, 1999; Rakshit et al. 2008). These findings support the use of PE-free accessions of Jatropha, as they give rise to byproducts (e.g., seed cakes) that can be used as a dietary source. Our results confirmed the metabolic equivalence of PE-containing and PE-free Jatropha accessions, except regarding PE biosynthesis. These findings demonstrate that PE-free accessions of Jatropha are safer than PE-containing accessions and suggest that the PE-free accessions are therefore likely to be more profitable in bioenergy applications.

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References

Adolf W, Opferkuch HJ, Hecker E (1984) Irritant phorbol derivatives from four Jatropha species. *Phytochem* 23: 129–132

El Diwani G, Attia NK, Hawash SI (2009) Development and

evaluation of biodiesel fuel and byproducts from jatropha oil. *Int J Environ Sci Technol* 6: 219–224

Goel G, Makkar HPS, Francis G, Becker K (2007) Phorbol esters: structure, biological activity, and toxicity in animals. *Int J Toxicol* 26: 279–288

Haas W, Sterk H, Mittelbach M (2002) Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. *J Nat Prod* 65: 1434–1440

Heller J (1996) Physic nut. *Jatropha curcas* L. Promoting the conservation and use of underutilised and neglected crops. I. Institute of Plant Genetics and Crop Plant Research, Gatersleben/International Plant Genetic Resources Institute, Rome

Hirota M, Suttajit M, Suguri H, Endo Y, Shudo K, Wongchai V, Hecker E, Fujiki H (1988) A new tumor promoter from the seed oil of Jatropha curcas L., an intramolecular diester of 12-deoxy-16-hydroxyphorbol. *Cancer Res* 48: 5800–5804

Makkar HPS, Aderibigbe AO, Becker K (1998) Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. *Food Chem* 62: 207–215

Makkar HPS, Becker K (1997) Potential of *J. curcas* seed meal as a protein supplement to livestock feed, constraints to its utilisation and possible strategies to overcome constraints. In: Proceedings the International Symposium on Biofuel and Industrial Products from *Jatropha curcas* and Other Tropical Oil Seed Plants; Managua, Nicaragua

Makkar HPS, Becker K (1999) Nutritional studies on rats and fish (carp Cyprinus carpio) fed diets containing unheated and heated Jatropha curcas meal of a non-toxic provenance. *Plant Foods Hum Nutr* 53: 183–192

Martínez-Herrera J, Siddhuraju P, Francis G, Dávila-Ortíz G, Becker K (2006) Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. *Food Chem* 96: 80–89

Ohta D, Shibata D, Kanaya S (2007) Metabolic profiling using Fourier-transform ion-cyclotron-resonance mass spectrometry. *Anal Bioanal Chem* 389: 1469–1475

Raja SA, Robinson smart DS, Lee CLR (2011) Biodiesel production from jatropha oil and its characterization. *Res J Chem Sci* 1: 81–87

Rakshit KD, Darukeshwara J, Rathina Raj K, Narasimhamurthy K, Saibaba P, Bhagya S (2008) Toxicity studies of detoxified Jatropha meal (*Jatropha curcas*) in rats. *Food Chem Toxicol* 46: 3621–3625

Singh RK, Padhi SK (2009) Characterization of jatropha oil for the preparation of biodiesel. *Nat Prod Rad* 8: 127–132

Singh A, Nigam PS, Murphy JD (2011) Renewable fuels from algae: an answer to debatable land based fuels. *Bioresour Technol* 102: 10–16

Stirpe F, Pession-Brizzi A, Lorenzoni E, Strocchi P, Montanaro L, Sperti S (1976) Studies on the proteins from the seeds of *Croton tiglium* and of *Jatropha curcas*. Toxic properties and inhibition of protein synthesis *in vitro*. *Biochem J* 156: 1–6