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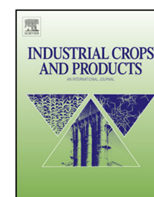

# Relative quantitation of ricin in *Ricinus communis* seeds by image processing☆

Ruwanthi C. Wettasinghe[a,b], Masoud Zabet-Moghaddam[b], Glen Ritchie[a], Dick L. Auld[a,*]

[a] *Department of Plant and Soil Science, Texas Tech University, Lubbock, TX, USA*
[b] *Center for Biotechnology and Genomics, Texas Tech University, Lubbock, TX, USA*

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ABSTRACT

Quantitative and qualitative assays for specific seed proteins could be used as a screening tool in breeding for improved seed characteristics. These assays could be especially useful for selection of castor seeds for altered expression of the toxin ricin. The technique described here includes extraction of water soluble proteins from partial seed samples so that the remaining seed is viable and can be used for genetic evaluation. Individual proteins are resolved by SDS-PAGE and identified by MALDI-TOF/TOF mass spectrometry. Once toxin proteins are correlated with bands in stained SDS-PAGE gels, image processing is used to estimate the relative quantities of these proteins in extracts from individual seeds. The results indicate that this method can serve as a relatively inexpensive and reliable assay for ricin content in castor endosperm tissues and this approach could facilitate the selection of low ricin castor varieties.

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## 1. Introduction

Castor seed oil is the primary commodity of *Ricinus communis* and is cultivated extensively throughout the world (Rivarola et al., 2011). Castor seeds contain up to 48–52% oil by seed weight. Similar to other oil seeds, castor seeds accumulate high levels of triacylglycerides (TAG) that function as food reserves for the early seedling growth (Huang, 1996). Castor oil also contains ricinoleic acid; an 18 carbon hydroxylated fatty acid with a single double bond, which is a unique raw material used in numerous commercial applications including lubrication, biodiesel, and plastic products. Castor seeds retain substantial endosperm at maturity and this tissue is the primary site of storage lipid accumulation (Wang et al., 2004).

The endosperm of developing castor seeds also accumulates storage proteins, which constitute between 10% and 40% of the seed dry weight (Shewry et al., 1995). The primary function of seed storage proteins is to provide essential amino acids to support protein synthesis during heterotrophic growth of the seedlings (Ahn and Chen, 2007). Some seed proteins play protective role; act as anti-fungal agents, protease inhibitors, and calmodulin antagonists (Pantoja-Uceda et al., 2003). Due to their abundance and economic importance, seed storage proteins were among the first plant proteins to be characterized. In 1924, T.B. Osborne classified seed proteins according to the solubility. These protein classes include (1) water soluble albumins, (2) globulins, which are soluble dilute saline, (3) alcohol/water soluble prolamines, and (4) glutelines, which are soluble in dilute alkyl/acid (Shewry et al., 1995). When characterized based on the sedimentation coefficients, the most abundant seed storage proteins in dicotyledonous species are 2S albumins, and 7S and 11S globulins (Otegui et al., 2006).

Lipids and proteins accumulate in oil bodies and protein storage vacuoles (PSV) respectively (Marshall et al., 2010). Castor seed PSVs contains phytin globoids and a single large protein crystalloid within a soluble protein matrix (Youle and Huang, 1976). The insoluble crystalloid is comprised of 11S globulins, while the soluble matrix is a mixture of 2S albumins and 7S lectins. The 7S lectins of castor seeds include ricin and *R. communis* agglutinin (RCA). Ricin is a potent cytotoxin with relatively weak hemagglutinin properties, while RCA is less toxic and exhibits powerful hemagglutinin properties (Chen et al., 2005). According to Barnes et al. (2009), ricin deposition in the developing castor seed does not occur until 28-days of post pollination. Ricin accumulates during seed maturation and capsule dehydration and continues to increase after germination of the mature seed until the 6th day after radicle emergence. The authors conclude that the primary purpose of ricin in seeds was to prevent seed predation during the quiescent stage.

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* Corresponding author at: Department of Plant and Soil Science, Texas Tech University, Food Technologies, Room 204A, Mailstop 2122, Lubbock, TX 79409-2122, USA. Tel.: +1 806 742 5704.
*E-mail address:* dick.auld@ttu.edu (D.L. Auld).

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The ricin gene encodes a 576 amino acid, 64.1 kDa, preproricin molecule (Audi et al., 2005). Removal of the signal sequence as preproricin enters the endoplasmic reticulum (ER) lumen forms a 61.6 kDa proricin. After the formation of an intramolecular disulfide bond to tether the A and B subunits of the mature heterodimer, proricin is subjected to vacuolar protease activity to form a 58.8 kDa mature ricin molecule. Due to its glycosidase activity the ricin A subunit acts as a protein synthesis inhibitor, while the B subunit functions as the recognition site for cell surface terminal galactose residues of glycoproteins and glycolipids (Peumans et al., 2001). Once ricin enters the cell, the A chain of the molecule can disrupt up to 1500 ribosomes per minute arresting cellular protein synthesis (Pinkerton et al., 1999).

Since reducing toxicity and allergenicity of castor seeds are key objectives of many castor breeding programs, efforts have focused on selecting accessions for divergent ricin and agglutinin concentrations (Morris et al., 2011). To meet this breeding objective, the semi-dwarf internode castor variety, Hale, was crossed with two accessions of castor PI 258368 and PI 257654, which were known to have reduced levels of ricin (Lowery et al., 2007). In subsequent segregating generations, individual plants were selected for semi-dwarf internode growth habit and reduced levels of ricin using a radial immune diffusion (RID) assay (Pinkerton et al., 1999; Auld et al., 2001, 2003). In 2003, twelve F8 lines were intercrossed to develop a broad genetic based synthetic population adapted to mechanical harvest. From 2004 to 2009, this experimental population was grown in isolation and was screened for semi-dwarf internode growth habit and reduced shattering. This process produced a new experimental castor variety, Brigham, which has seven- to ten-fold reductions in ricin levels relative to the semi-dwarf internode Hale parent (unpublished data Dr. Xiaohua He). However, due to the intercrossing of 12 parent lines for six cycles it was thought that there could be a significant variation of ricin content among individual plants as well as within the seeds of a single plant. In 2010, two hundred individual plants of this Brigham variety were harvested from the isolated seed increases in Pecos, Texas.

The purpose of this study was to develop and rapid and inexpensive method to identify and select castor (*R. communis*) *var.* Brigham seeds of half sib families that are low in ricin content. This objective was accomplished by SDS-PAGE analysis of partial seed samples coupled with MALDI-TOF/TOF protein identification and image analysis.

## 2. Materials and methods

### 2.1. Plant material

Open pollinated half-sib castor seeds (*R. communis* L. *var.* Brigham) were harvested from approximately 200 plants grown in Pecos, Texas in 2010. Seeds were characterized based on their oil content, seed weight, percent mesocarp, and mesocarp weight. Seeds of 50 plants were selected based on the highest oil content and the lowest mesocarp retention. Of these fifty plants, the seeds of nine randomly selected plants were analyzed in this study. In addition, seeds of *R. communis* L. *var.* Hale were used as a parental check. Each seed was dissected using a razor blade to harvest section consisting of approximately 1/3 of the seed most distal to the caruncle. This section has a higher average ricin content compared to the other 2/3 of the seed (Pinkerton et al., 1999).

### 2.2. Soluble protein extraction

Six extraction solutions were prepared and compared for their suitability to extract water soluble proteins of Hale seed sections. The compositions of solutions used to extract soluble proteins are listed in Table 1. The efficacy of above extraction solutions were compared by preparing equivalent masses of six sets where each set comprising three endosperm tissue sections of *var.* Hale seeds. Using a clean razor blade, the top one-third sections of three seeds excised, the seed coats removed, and the resulting tissue sample weighed. Each set was transferred to a separate mortar placed on ice, 5× (v/w) of each grinding solution was added and homogenized using a pestle kept at 4 °C. Each homogenate was transferred into 1.5 mL tubes and were centrifuged at room temperature for 10 min at 10,000 × *g*. The liquid phase (crude extract) was transferred carefully into a 1.5 mL tube. One hundred micro-liters of each crude extract were transferred into a 1.5 mL tube and mixed with 400 μL of 100% acetone at −20 °C. Samples were incubated at −20 °C for 1 h and 30 min and then centrifuged at room temperature for 10 min at 32,066 × *g*. The liquid phases were discarded, the remaining pellets were air dried for 3–5 min, and dissolved in appropriate volumes of re-solubilization solution containing 0.1 M Tris pH 6.8, 1% SDS. If needed, the resolubilized pellet was stored at −20 °C until further use. In addition, twelve seeds comprised of two more seeds of *var.* Hale (H1, and H2) and three seeds from 3 half sib families (18-3, 18-4, 18-5, 90-3, 90-4, 90-5, 119-3, 119-4 and119-5) were selected randomly from *var.* Brigham and compared by analyzing soluble protein profiles of each seed. The seed excision technique was similar to the method described previously and extraction solution 5 was used. Re-solubilization of the extracted pellet and storage of extracted proteins was carried out as described above. The protein concentrations of the samples were determined using Bio-Rad Protein Assay™ (Bio-Rad; Hercules, CA), based on a Benchmark Plus™ microplate reader (Bio-Rad; Hercules, CA) according to manufacturer's instructions.

**Table 1**
Solution identifications and compositions used in soluble protein extraction of castor endosperm tissues.

| Solution ID | Composition |
|---|---|
| 1 | 0.1 M Tris pH 6.8, 1% SDS, 1× protease inhibitor[a] |
| 2 | 0.1 M Tris pH 6.8, 1% SDS |
| 3 | 0.1 M Tris pH 6.8, 1× protease inhibitor[a] |
| 4 | 0.1 M Tris pH 6.8 |
| 5 | Distilled water |
| 6 | Distilled water, 1× protease inhibitor[a] |

[a] Complete EDTA-free™ cocktail tablets [Roche; Basel, Switzerland] prepared according to manufacturer's instructions.

### 2.3. Protein separation visualization and quantitation

Protein samples were diluted in double distilled water to obtain desired concentration and incubated at 70 °C for 10 min in the presence of 1× NuPAGE LDS™ sample buffer (Invitrogen; Carlsbad, CA). All samples were prepared under non-reducing conditions to maintain disulfide linkages. The NuPAGE apparatus (Invitrogen; Carlsbad, CA) was assembled and 1X SDS running buffer was prepared according to manufacturer's recommendations. Samples were loaded (2–16 μg per well) into pre cast 1 mm thick 4–12% NuPAGE® Novex® Bis–Tris separation gel with 4% stacking gel (Invitrogen; Carlsbad, CA). SeeBluePlus2® (Invitrogen; Carlsbad, CA) pre-stained protein marker was loaded as the molecular size standard. The gel was run at a constant voltage of 200 V until the bromophenol blue loading dye migration was within 1 cm of the bottom of the gel. The gel was removed from its outer cassette, stained in blue-silver Coomassie stain at room temperature for 12 h, and destained in double distilled water. The destained gel was photo-documented using the automated Molecular Gel Doc XR+® (Bio-Rad; Hercules, CA) system and then the distinct bands of the gel were quantified using Image Lab ver.2® (Bio-Rad;

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