EVALUATION OF RESISTANCE OF KENYAN SUNFLOWER TO LEAF TISSUE DEGRADATION BY OXALIC ACID AND THE OIL YIELD

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Abstract
Oxalate oxidase is a pathogenesis-related enzyme that occurs naturally in plants and whose activity is observed to increase when hosts are attacked by oxalic acid-producing pathogens, countering the effects of the acid and thus conferring resistance to infection. This study, through two assays, sought to investigate the level of oxalate oxidase activity in six Kenyan sunflower (Helianthus annuus L.) varieties namely: Kenya Fedha, Rekord, Issanka, H8998, H4038 and H4088. The reaction of oxalate oxidase with its substrate, oxalic acid, yields hydrogen peroxide which can be assayed calorimetrically as a measure of enzyme activity. In this study, an assay which focused on release of hydrogen peroxide from sunflower leaf discs incubated in a buffer containing oxalic acid was conducted. A detached leaflet assay was also conducted to assess the ability of oxalate oxidase to prevent necrosis in response to exogenous application of oxalic acid to plant tissue whereby lesion sizes were measured. The oil content of the six sunflower varieties was also determined to identify the ones with the highest oil-yielding potential. The test sunflower varieties differed significantly ($p<0.05$) in oxalic acid degradation and in their hydrogen peroxide production, indicating varying degrees of oxalate oxidase activity. H4088 and H8998 had the highest seed oil content and were identified as superior sunflower varieties that could be recommended for edible oil production in Kenya. However, H8998 may require genetic transformation to enhance its disease resistance capacity.

Key words: Oxalate oxidase, oxalic acid, sunflower

Introduction

Sunflower *(Helianthus annuus L.)* is an important annual crop grown in Kenya for its edible oil contained in the seeds. Production has decreased over the years due to challenges such as pests and diseases (Okoko *et al.,* 2008), leading to huge imports of seed and crude edible oil to meet domestic demand. To ameliorate this shortage, the search for disease resistant varieties that are also high yielding remains imperative.

Plants defend themselves against disease-causing pathogens by a combination of weapons from two arsenals, structural and biochemical defenses. Biochemical defenses are reactions that take place in the plant cells and tissues and produce substances that are either toxic to the invading pathogen or that create conditions that inhibit growth of the pathogen in the plant (Agrios, 2005).

Pathogenesis-related (PR) proteins are a structurally diverse group of proteins found in trace amounts in plant and are toxic to invading fungal pathogens. Their accumulation is observed in many plant species upon stress or infection by pathogens and result in acquired host resistance against the invading pathogens (Stintzi *et al.*, 1993).

Oxalate oxidase (OXO) is a PR protein that belongs to the germin family of proteins. It catalyzes the degradation of oxalic acid to carbon dioxide and hydrogen peroxide (Chiriboga, 1966; Dickman and Mitra, 1992). OXO activity has been reported in a number of cereals such as maize, wheat, oat, rye and barley in response to attack by oxalic acid-producing pathogens (Dunwell *et al*., 2000). The enzyme's potential role in fighting fungal infection has led to much effort towards the generation of transgenic plants with higher oxalate oxidase activity and resistance to infection. Wheat has a high innate oxalate oxidase activity (Dunwell *et al.,* 2000) and was therefore used as the control in this study.

Hydrogen peroxide (H_2O_2) is naturally produced by plants at low concentrations (Chen *et al.*, 1993) and is involved in a wide variety of beneficial reactions and signaling cascades necessary for all aspects of plant growth and metabolism. Chen reported concentrations of 0.15 µmol/g fresh weight ($gFW^{-}1$) in stress-free tobacco leaves, 0.35 µmol/ $gFW^{-}1$ in stress-free pear fruit and 0.067 µmol/$gFW^{-}1$ in stress-free mangrove.

Hydrogen peroxide is however produced in relatively higher amounts during plant stress responses thus enhancing resistance mechanisms. Studies by Chen revealed 10-fold higher tissue H_2O_2 levels than the controls after 24 h exposure of plants to acute stress. In plant stress studies, plants are grown in controlled conditions, and a stress applied rather suddenly after a period of unstressed growth. Some aspect or aspects of response – ranging from activity of a single enzyme to whole genome transcript networks – at a fixed time thereafter -are then analyzed (Cheeseman, 2007).

Complete resistance of cultivated sunflower to oxalic acid-producing pathogens has not been reported but significant levels of resistance have been identified in diverse germplasm (Nelson and Lamey, 2000; Rashid and Dedio, 1992). Resistance of

Kenyan sunflower varieties to oxalic acid degradation has not been investigated. This study aimed at determining oxalate oxidase activity in selected sunflower varieties and their seed-oil content in order to identify the ones that have potential for recommendation for edible-oil production in Kenya.

Materials and Methods

Seeds of six sunflower varieties namely Issanka, Rekord, Kenya Fedha, H8998, H4088 and H4038 were obtained from Kenya Agricultural Research Institute (KARI) and Kenya Seed Company while wheat seeds, variety Kwale, were obtained from Kenya Seed Company.

Planting of Sunflower and Wheat Seedlings

To ensure good water holding capacity, porosity and drainage, the soil medium used was prepared by mixing loam soil, compost and sand in the ratio 3:2:1 as described by Bunt (1976).

Seedlings were supplemented with nitrogen, phosphorous, potassium and micronutrients by application of a foliar feed (Bayfolan, Bayer Crop Science, Germany) as prescribed.

Seventy five seeds of each sunflower variety were planted (fifteen seeds of each variety, every week, for five weeks) in plastic polythene bags in a greenhouse at Jomo Kenyatta University of Agriculture and Technology. After germination, the seedlings were watered to field capacity. Thirty wheat seeds were also planted in similar polythene bags (five seeds in each bag, every week) and grown in the same greenhouse as the sunflower seedlings. Both wheat and sunflower leaves were randomly harvested 1½ months after planting for use in various laboratory analyses. This procedure was carried out for two consecutive seasons.

Oxalic Acid Bioassay

Leaflet assays were conducted to assess the ability of oxalate oxidase to prevent damage in response to exogenous application of oxalic acid to plant tissue according to the modified method of Livingstone *et al*. (2005). Detached sunflower leaflets were arranged on 12-cm Petri dishes containing dampened paper towels. Eighteen leaflets were used for each variety as three replicates for each concentration of oxalic acid. Oxalic acid concentrations ranged from 0-10 mM. Each leaflet was wounded in four locations on the abaxial surface with an 18-gauge needle and 15 µL oxalic acid applied to each wound. Leaflets were incubated for 48hrs at room temperature (25 ± 2^{o}C). To measure lesion size, leaflets were washed with distilled water and viewed with a dissecting microscope (SZ ZT, Olympus, Japan). Triplicates of wheat (control) leaflets per concentration of oxalic acid were also wounded as the sunflower leaflets and 15µL of oxalic acid applied on the wounds. Lesion areas on wheat leaflets were compared against those on sunflower leaflets based on a 5-point assessment scale (Table 2).

Oxalate Oxidase Assay

The assay focused on the release of hydrogen peroxide from sunflower leaf tissue as a measure of oxalate oxidase activity and was conducted according to the method of Sugiura *et al*. (1979). To determine oxalate oxidase activity, leaf discs (5mm diameter) were incubated in 1.5ml microfuge tubes with 200µl of assay buffer (18mg oxalic acid in 100ml of 2.5mM succinic acid, pH 4) and reactants incubated for 15 min at 37^{o} C. After incubation, developing solution (135µl) was added to the tubes and the reaction allowed to continue at room temperature (25 ± 2^{o}C) for 30 min. The developing solution, which consisted of 6mg of aminoantipyrene dissolved in 30µl of *N, N*-dimethylaniline, was then added to 100ml of 0.1M sodium phosphate buffer, pH 7.0 containing 57µl of a 140mg ml^{-1} solution of horseradish peroxidase. The contents of the microfuge tubes were diluted ten times prior to measurement of absorbance at a wavelength of 550nm using a UV-Visible spectrophotometer (UV-1601 PC Shimadzu Corp, Japan). The wheat control and blank (buffer without leaf discs) were given the same treatment as the samples. A plot of hydrogen peroxide standards in the range of 0-10mM was used to quantify the hydrogen peroxide produced by the leaf discs.

Determination of Oil Content

Ten grams of ground sunflower seed of each variety were put in an extraction thimble and total lipids extracted by the Soxhlet Method (AOAC, 1980) using 150ml of petroleum spirit for 16hr at 80^{o}C. The oil was dried in an oven at 105^{o}C for one hour, cooled in a dessicator and percentage oil content calculated on a weight by weight basis.

Results and Discussion

In this study, plants were grown in a controlled green house environment to ensure they were stress-free. Subjecting the leaf tissue to stress either by exogenously applying or incubating the leaf tissue in oxalic acid elicited production of hydrogen peroxide whose concentration was determined as a measure of oxalate oxidase activity. As earlier stated, oxalate oxidase is known to break down oxalic acid to carbon dioxide and hydrogen peroxide.

Direct application of oxalic acid to both wheat and sunflower leaf tissue caused formation of necrotic lesions whose size was observed to increase with increasing concentration of oxalic acid (Figure 1). There were significant differences ($p<0.05$) in mean lesion areas per variety (Table 1). Degradation of Kenya Fedha, Issanka and Rekord by oxalic acid was high while that of H4038, H8998 and H4088 was intermediate (Table 2). The level of degradation in wheat was low clearly supporting the protective role of its innate oxalate oxidase against leaf tissue degradation by oxalic acid. On the contrary, the larger lesion areas observed in sunflower indicated that the amount of hydrogen peroxide produced was low, which directly translated to low oxalate oxidase activity that is unlikely to play a major protective role in sunflower defense against oxalic acid degradation, and by extension, to oxalic acid-producing pathogens.

In the oxalate oxidase assay, the activity of oxalate oxidase was measured spectrophotometrically by determining the amount of hydrogen peroxide produced from the breakdown of oxalic acid. The hydrogen peroxide produced was enzymatically broken down by horse radish peroxide, a process accompanied by oxidative coupling of aminoantipyrene with *N, N*-dimethylaniline. The resulting indamine dye was determined colorimetrically at 550nm as a measure of H_2O_2 concentration using H_2O_2 standards.

There was a significant difference between the mean hydrogen peroxide production by the control and test varieties ($P<0.05$) where wheat leaf discs produced up to 2.74 times more H_2O_2 compared to sunflower leaf discs (Figure 2), indicating a higher oxalate oxidase activity. This observation correlated well with that of the detached leaflet assay which showed wheat to be more resistant to oxalic acid degradation and thus a higher oxalate oxidase activity than sunflower at concentrations used in this study. This study also concurs with that of Hu *et al.* which demonstrated that sunflower transformed with a wheat oxalate oxidase gene demonstrated increased resistance to *S. sclerotiorum,* an oxalic acid-producing pathogen.

Within the sunflower varieties, the mean H_2O_2 production was also significantly different ($p<0.05$), most probably due to differences in expression levels of oxalate oxidase (Livingstone *et al*., 2005). As expected, the higher amount of hydrogen peroxide produced by H4088 and H4038 (Figure 1), correlated positively with their detached leaflet assay values (Table 1), which displayed a relatively low degree of degradation, closely followed by H8998. Similarly, Kenya Fedha, Issanka and Rekord showed low levels of enzyme activity and high levels of degradation at concentrations of oxalic acid used in this study.

The 48 hour period that was used in this study covered the critical phases when plant responses determine the ultimate success or failure of attempted infection or when fungal ascospores or mycelia initiate their attack on leaf epidermal cells (Helene *et al*., 2000). The pH of 4 used in the enzyme assay is also the optimum pH for the fungal polygalacturonase activity (Cessna *et al*., 2000), suggesting synergism between oxalate secretion and pectin degradation.

There was a significant difference ($p<0.05$) in oil yield among the varieties with H8998 recording the highest seed oil content (50.55% w/w) on dry weight basis (Figure 3). The varieties H4088, Rekord, Kenya Fedha, Issanka and H4038 yielded 49.41%, 47.66%, 41.60%, 41.21% and 40.92% (w/w) seed oil content, respectively, on dry weight basis.

Conclusion and Recommendations

The efficacy of constitutively expressed oxalate oxidase in enhancing resistance of sunflowers to the degrading effects of oxalic acid was clearly demonstrated in this study by using wheat as control.

Kenyan sunflower varieties showed low resistance to the effects of exogenously applied oxalic acid compared to wheat, and thus the likelihood of low resistance to oxalic-acid producing pathogens.

No direct relationship was found between the oil yield and oxalate oxidase activity (Figure 3). The variety H4088 was identified as the most resistant to oxalic acid degradation among the test varieties with relatively high seed oil yield. This is an important finding that is expected to encourage sunflower cultivation in Kenya. Its promotion will ultimately lead to enhanced vegetable oil security in the country.
The variety H8998 was found to have low resistance but with the highest oil content and is thus recommended for resistance improvement using a resistance gene to enhance its oxalate oxidase activity.
This study further recommended that investigation of resistance using natural pests such as *Sclerotinia sclerotiorum* be carried out in the six varieties under study, and that molecular studies be conducted to determine their genetic potential for fungal pest resistance.

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Appendices

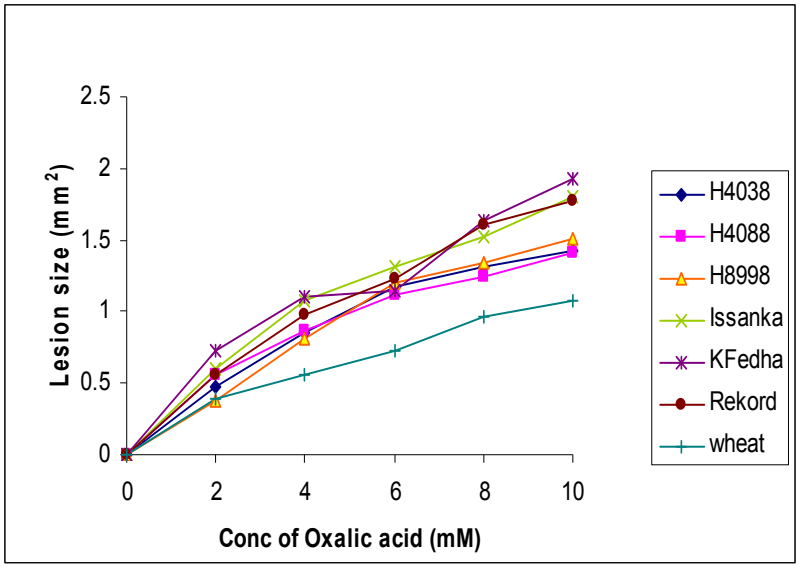

Figure 1. General increase in lesion areas following application of increasing concentrations of oxalic acid.

Table 1. Mean lesion areas (mm^2) following degradation of leaf tissue by increasing concentrations of oxalic acid

Variety	**Concentration of oxalic acid (mM)**					
	0	2	4	6	8	10
H4038	0	0.48	0.85	1.18	1.31	1.43
H4088	0	0.56	0.87	1.12	1.24	1.41
H8998	0	0.37	0.81	1.20	1.34	1.50
Issanka	0	0.59	1.08	1.31	1.52	1.80
K. Fedha	0	0.73	1.10	1.14	1.63	1.92
Rekord	0	0.56	0.98	1.22	1.60	1.77
Wheat	0	0.39	0.56	0.73	0.97	1.00

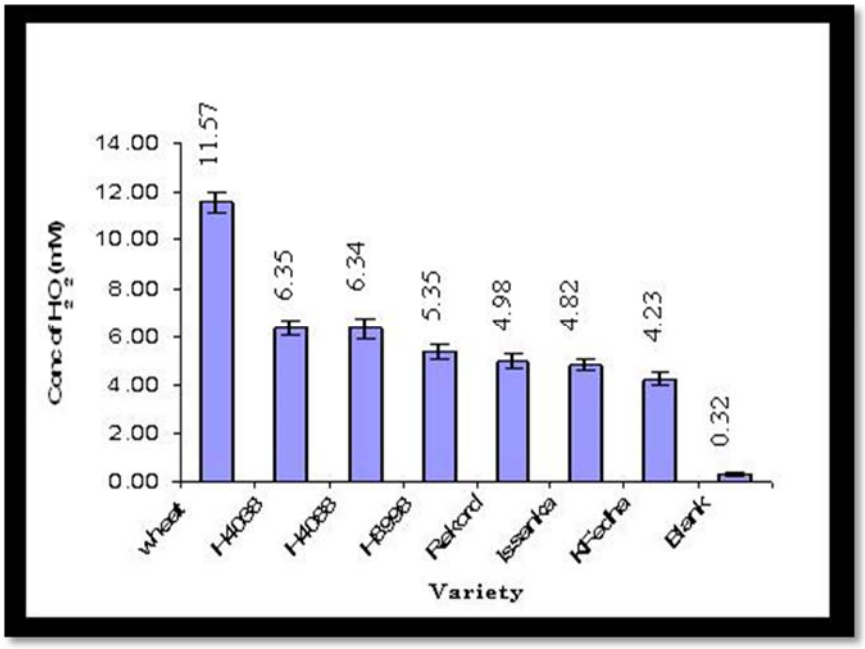

Figure 2. Concentrations of H_2O_2 (mM) generated from leaf discs of wheat and different sunflower varieties. Hydrogen peroxide content was determined spectrophotometrically at 550 nm

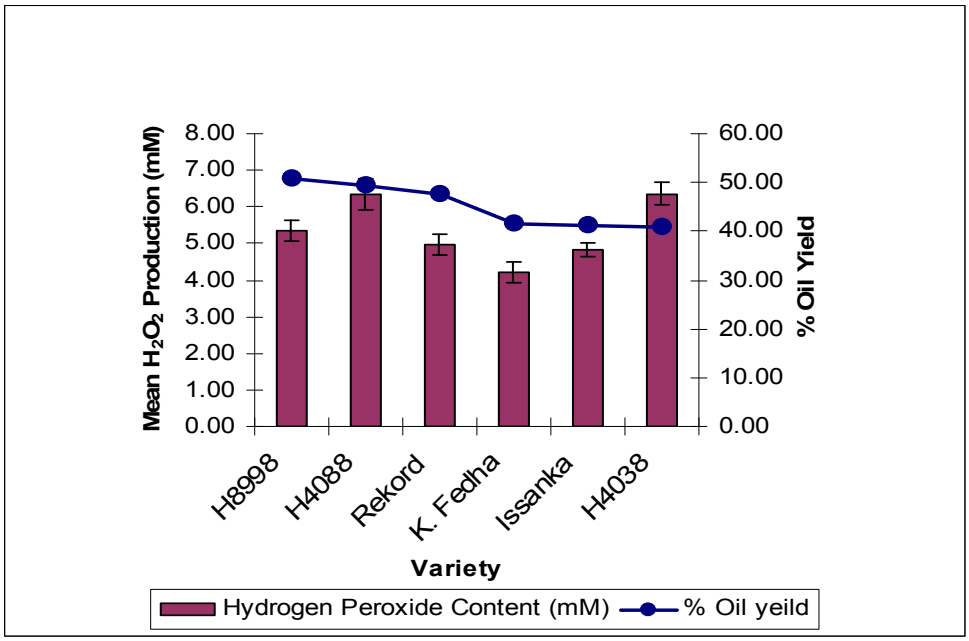

Figure 3. Relationship between oxalate oxidase activity and oil yield

Table 2: Assessment scale for lesion development

Lesion size (mm^2)	Degradation level
0.00 - 0.50	Extremely low
0.51 - 1.00	Low
0.01 – 1.50	Intermediate
1.51 – 2.00	High
2.01 – 2.50	Extremely high