Production of Free Fatty Acids in Safflower Seeds by Fungi[1]

T.C. HEATON, P.F. KNOWLES, D.S. MIKKELSEN, and J.E. RUCKMAN,
Department of Agronomy and Range Science, University of California, Davis, California 95616

ABSTRACT

In 1975, free fatty acids (FFA) reached 0.50% in hand-sampled seeds (achenes) from the Sacramento Valley but never exceeded 0.10% in samples from the San Joaquin Valley. High levels of FFA in safflower seeds from the Sacramento Valley were attributed to seed-borne fungi, especially *Alternaria* sp., having a lipolytic action on safflower oil. Greenhouse inoculations on representative safflower cultivars reduced yields and oil content while increasing FFA. In vitro experiments using safflower oil-potato dextrose agar medium showed that the *Alternaria* species and four other fungi isolated from safflower had lipolytic activity. The *Alternaria* sp. produced 3.90% FFA after 20 days of growth on this medium. Separation of the FFA by thin layer chromatography and characterization by gas liquid chromatography indicated that fatty acids are released by all five fungi in proportions similar to those in safflower oil.

INTRODUCTION

Identification of the causes of high levels of free fatty acids (FFA) in vegetable oils has been a goal of recent research since the reduction of FFA would considerably reduce refining losses. Lipolytic enzyme systems have been found in most oilseeds examined to date (1,2). Most seed lipases remain inactive in intact seeds and are activated by either germination or mechanical damage. Care in the harvest, storage, and processing of seeds can prevent seed lipases from producing high FFA in oil.

Acidity in oils has been attributed also to fungi with lipolytic capabilities. *Aspergillus* sp. produced high FFA in chocolate when cocoa beans were artificially inoculated (3). Similarly, a lipase from a strain of *Aspergillus flavus* degraded coconut oil (4). A strain of *Helminthosporium maydis* Race T readily used corn oil as a substrate, and lipolytic activity of the fungus increased FFA (5). *Aspergillus flavus* on cottonseed following a preharvest rain produced high FFA in cottonseed oil (6). Rainy weather likewise favored the spread and development of sunflower diseases which impaired oil quality (7). *Sclerotinia sclerotiorum* and *Botrytis cinerea* decreased oil content and increased FFA content. Sunflower seeds (achenes) with thin hulls had higher FFA than seeds with thick hulls. *Rhizopus* sp. caused hull and embryo discoloration in sunflower (8), and the authors speculated that oil quality was lowered.

In recent years processors of California-grown safflower seeds (achenes) have noted increasingly higher FFA in safflower oil ($>$ 0.30% as oleic acid compared to previous levels near 0.10%). When FFA exceed 0.30%, the efficiency of deodorization without prior caustic refining is considerably reduced. Preliminary data suggested that freshly harvested seeds from specific locations in the Sacramento Valley had higher FFA. This paper discusses the causes of higher FFA in safflower oil.

MATERIALS AND METHODS

Seed Samples

During the 1975 cropping season safflower seed samples were collected from commercial fields in the San Joaquin and the Sacramento Valleys of California. Plants were sampled by hand before harvest, and the safflower heads were hand-threshed to avoid seed damage. To measure the effect of mechanical damage on FFA, samples were taken also from combine harvest at the same locations. Combine and hand samples were stored in sealed canning jars at 4 C to retain moisture and prevent seed deterioration. Samples were analyzed for fungal infection, FFA, moisture, and oil contents.

Greenhouse Studies

Plants grown in the greenhouse were inoculated at full bloom with a spore suspension of *Alternaria* sp. isolated from grower seed samples. The suspension was prepared by scraping spores from cultures grown on potato-dextrose agar in petri dishes. The surface of the culture was rinsed with distilled water followed by filtration through several layers of cheesecloth. After spore suspensions were applied with an atomizer, the treated plants were covered with plastic bags to retain moisture and promote infection. Control plants were sprayed with distilled water and covered in the same manner. After 36 hr the bags were removed and the plants allowed to mature normally. Mature heads were collected from control and inoculated plants. FFA content, oil content, and yield (grams of seed/head) were determined.

Seed Quality Measurements

The FFA content of all samples was determined by modification of the AOCS Official Method Cd 3a-63. Safflower oil was expelled from seeds with a Carver press. Approximately 5 g of oil were dissolved in 100 ml of isopropanol and toluene (1:1, v/v) previously neutralized with 0.1N KOH. The mixture was titrated with 0.1N KOH to a phenolphthalein end point. FFA content was calculated as the percentage of oleic acid.

All samples tested for moisture content had been stored in sealed canning jars at 4 C. Preweighed samples were dried in a forced-draft oven at 120 C for 4 hr, and moisture content was calculated.

Seed oil content was determined with a Nuclear Magnetic Resonance Analyzer (Newport of North America) on seed dried at 60 C for 24 hr.

For seed fungal infection, seeds were sampled at random, surface-sterilized for 15 min in a 1:10 dilution of commercial bleach, and then rinsed in sterile water. They were then placed on a sterile 10% NaCl potato-dextrose agar medium in petri dishes. The petri dishes were incubated under fluorescent lights at room temperature. After 3 days the number of seeds showing fungal growth and the fungal species were noted. The assay was replicated twice for each sample.

Storage Studies

Safflower with normal hulls (variety Gila) was chosen for storage studies. To measure only the effects of damage and moisture content on FFA production, the seeds were taken from the San Joaquin Valley where fungal contamination was negligible. Intact and artificially damaged seeds (18 to 20% hull damage by gently tapping seeds with a hammer) from the same source were placed on suspended screens in five divided desiccators according to the method of Frey and Hammond (9). After 2 months of storage the

[1] Presented in part at the AOCS Meeting, New York, May 1977.

TABLE I

Summary of FFA and Seed Infection by Fungi in Safflower Samples Collected in the San Joaquin and Sacramento Valleys, 1975

Location	Number of samples[a]	Average % FFA[b]	% FFA range	% Infected seed range
San Joaquin Valley[c]	22	.064 A	.032-.094	0-6
Freeport to Ryer Island	4	.085 ABC	.048-.147	26-92
Davis	20	.143 BCD	.052-.449	12-100
Yolo Bypass to Sutter Basin	6	.088 ABCD	0.36-.136	26-100
North Yolo County	17	.152 CDE	.042-.546	85-100
Glenn County	20	.159 E	.084-.258	not available

[a]Samples were grouped without varietal distinction.

[b]Analysis of variance and pairwise LSDs were determined on power-transformed data according to Hinz and Eagles (11). Means followed by the same letter are not significantly different at the 0.05 level.

[c]All other locations are in the Sacramento Valley.

TABLE II

Differences in FFA of Combine and Hand Samples from Nine Commercial Fields in 1975[a]

Sample	Number of samples	FFA % Average	FFA % Range
Combine	9	.137	.034-.300
Hand	9	.103	.036-.119
	t = 1.349 n.s.		

[a]Paired *t*-test.

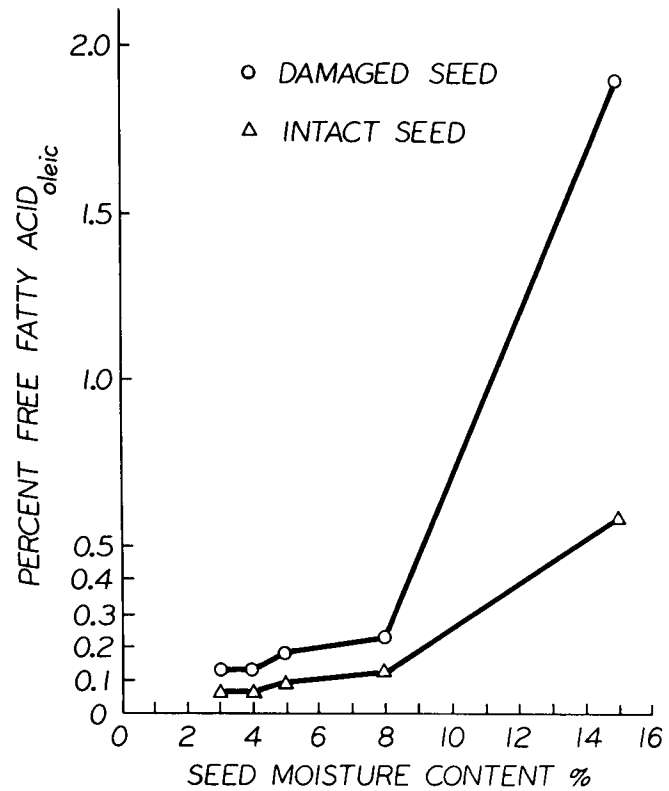

FIG. 1. Effect of different moisture contents on FFA levels in damaged and intact safflower seeds after 2 months storage.

moisture content for damaged and undamaged seeds in each desiccator was determined. FFA content was measured in oven-dried samples of both types of seeds.

Fungal Studies

Five species of fungi, one from each of the genera *Alternaria, Aspergillus, Botrytis, Rhizopus,* and *Stemphylium,* were tested for their ability to degrade safflower oil into FFA, glycerol, and partial glycerides. The *Alternaria* sp., *Rhizopus* sp., and *Stemphylium* sp. were isolated from safflower seeds taken from commercial fields in 1975. *Aspergillus* sp. was found on safflower seeds stored in a warehouse for 10 days. The *Botrytis* sp. was obtained from a culture maintained by the USDA Agricultural Research Service in the Department of Plant Pathology, University of California, Davis. It had been isolated previously from blighted safflower heads. Each fungus was grown in nine flasks on sterile 25% safflower oil-potato dextrose agar (PDA) medium. Fifteen other flasks containing only PDA were also inoculated, three flasks for each fungus, to determine whether acidity in the medium was due to acidic fungal metabolites. Controls consisted of nine uninoculated flasks containing safflower oil-PDA medium to assess the effect of PDA on safflower oil breakdown over time. Incubation was at room temperature under 100-watt incandescent lights. It was noted that incandescent light promoted mycelial growth, whereas fluorescent light favored sporulation.

At intervals of 5, 10, and 20 days, one-third of the flasks from each of the three treatments (safflower oil-PDA medium with and without fungi, and PDA medium with fungi) were evaluated for increased FFA. The safflower oil was extracted from the media by adding 50 ml of petroleum ether and liquefying the contents of the flasks with an Aero-mixer. The mixture was filtered through S&S 100 No. 560 filter paper into a 100-ml beaker, and the petroleum ether was evaporated from the filtrate and FFA was determined on recovered oil. The inoculated medium with only PDA was extracted similarly.

FFA were separated by thin layer chromatography from safflower oil on which fungi had grown for 20 days. The oil was applied in a band on 20 x 20-cm glass plates coated with 0.625 mm of Silica Gel G. A solvent system of petroleum ether, diethyl ether, and acetic acid (85:15:1, v/v) was used. Lipid classes were located by spraying the plates with 2,7-dichlorofluorescein and viewing under UV light. The FFA region was scraped from the plate, and the FFA were eluted from the absorbent with 10 ml of diethyl ether.

After evaporation of the diethyl ether, methyl esters of the FFA were made by adding 3 to 5 ml of 1.25% thionyl chloride in dry methanol (1:80 mixture, $SOCl_2$:methanol) and refluxing gently for 30 min. The remainder of the esterification mixture was evaporated, and the methyl esters were taken up in 0.5 ml of hexane (10).

Methyl esters of the FFA were quantified by gas liquid chromatography (10). Fatty acids were identified by known standards. Fatty acids of safflower oil were esterified in the same manner and were compared by Chi-square analysis with the FFA produced by the fungi.

TABLE III

Data from Inoculations of Safflower Plants with *Alternaria* sp. in a Greenhouse

Variety	Inoculated	Control
	Seeds per head, g	
S-208	0.80	0.99
S-296	1.07	1.41
Pacific 1	0.99	1.40
658-12	0.71	0.90
Mean	0.89	1.18
t = 5.29[a]		
	Oil content, %	
S-208	33.6	37.1
S-296	33.3	41.0
Pacific 1	32.7	35.5
658-12	40.4	41.4
Mean	35.0	38.8
t = 3.36[a]		
	FFA as oleic, %	
S-208	3.132	0.100
S-296	3.360	0.061
Pacific 1	3.513	1.175
658-12	1.307	0.073
Mean	2.828	0.352
t = 5.37[a]		

[a]Indicates significant difference between inoculated and control plants at the 0.05 level. Paired *t*-test.

RESULTS

Origin of FFA

Table I summarizes the results from sampling done in 1975. Higher FFA was found more frequently in samples from the Sacramento Valley than in those from the San Joaquin Valley. Each location in the Sacramento Valley had a wide range of FFA values. Oil content was independent of FFA value, as expected (data not presented). Moisture content at harvest time for both locations was near 5.0% on the average, although higher percentages were found at some Sacramento Valley locations (data not shown). The highest moisture content was 12.9% – from the North Yolo County sample in the Sacramento Valley that also had the highest FFA content, 0.546%. Other samples with high FFA had normal moisture contents, however, indicating that abnormal moisture is not directly related to high FFA.

FFA content did not differ significantly between combine samples and hand samples (Table II). This suggests that normal seed damage at harvest (<10% damaged seeds) does not immediately affect FFA levels. However, Figure 1 shows that seed damage in combination with high moisture content during storage will eventually increase FFA above acceptable levels.

Since most samples with high FFA usually had normal moisture contents, were not excessively damaged, and had not been stored for more than 1 week, FFA in safflower oil must be due to external factors. It was noted during sampling that the foliage of safflower plants from many locations in the sacramento Valley was gray to almost black. The dark appearance was presumably due to saprophytes on the leaves. Plants in the San Joaquin Valley were a clean golden yellow. Cultured seeds from samples showed that the incidence of seed-borne fungi also increased from the San Joaquin Valley to the Sacramento Valley (Table I). When seed infection was found, an *Alternaria* sp. consistently predominated in culture dishes. Species of *Aspergillus, Botrytis, Fusarium, Rhizopus,* and *Stemphylium* were found in isolation frequencies of less than 5% of the total infection.

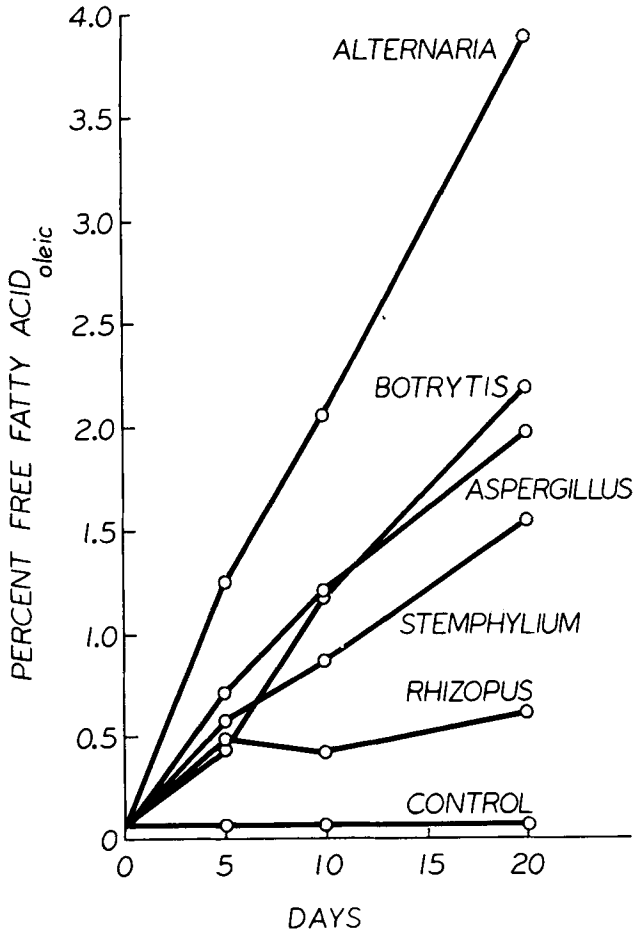

FIG. 2. FFA in safflower oil produced by fungi grown on safflower oil-PDA medium.

Because high FFA in seeds from the Sacramento Valley was obviously associated with the seed-borne *Alternaria* sp., plants in the greenhouse were inoculated with that fungus. Table III shows that inoculations of the *Alternaria* sp. on four representative safflower cultivars grown in the greenhouse reduced yield by 25%, and oil by 10%, and raised FFA to 8 times that in control plants. Seeds from inoculated plants were 100% infected.

In Vitro Production of FFA

Figure 2 shows increases in acidity in safflower oil-PDA medium on which *Alternaria* sp. and four other common fungi were grown. All of the fungi were capable of increasing the FFA content of safflower oil. Within 5 days, all had increased the FFA in recovered oil to undesirable levels (0.30% as oleic acid). The *Alternaria* sp. had an extraordinary ability to cause acidity in safflower oil. The *Rhizopus* sp., had the lowest FFA production over the 20-day period.

It is unlikely that increased acidity in the safflower oil-PDA medium was caused by acidic metabolites, for fungi grown on an oil-free medium did not produce acidity. Likewise, the uninoculated safflower oil-PDA medium did not increase in acidity.

Separation and characterization of the FFA caused by the five fungi after 20 days of growth revealed that the fungi attacked the safflower triglycerides without specificity for individual fatty acids. Table IV shows that the FFA released by the fungi were not significantly different in proportions from the fatty acid composition of safflower oil. These results suggest that these fungi act enzymatically on safflower oil to produce FFA.

TABLE IV

Comparison of the Fatty Acid Composition of Safflower Oil with the FFA Produced by Fungi Grown 20 Days on Safflower Oil-PDA Medium

Source	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	χ^2	P
Safflower oil	10.4%[a]	2.7%	12.4%	74.5%		
Alternaria sp. FFA	9.6	3.4	12.6	74.4	.252	.95-.99
Aspergillus sp. FFA	13.9	2.8	10.7	72.6	1.463	.50-.95
Botrytis sp. FFA	15.4	2.7	10.3	71.6	2.872	.30-.50
Rhizopus sp. FFA	13.8	4.8	13.2	68.2	3.329	.30-.50
Stemphylium sp. FFA	13.4	6.0	12.2	68.4	5.401	.10-.20

[a]Means of duplicate analyses.

DISCUSSION

The geographic occurrence of FFA in safflower oil in this study is analogous to the situations in cottonseed and sunflower reviewed in the Introduction. The pattern of FFA occurrence coincides with the incidence of seed-borne fungi, especially *Alternaria* sp. This fungus is probably more damaging in the Sacramento Valley than in the San Joaquin Valley because of the more favorable environment. *Alternaria* is ubiquitous there, living on organic debris or in weedy areas surrounding fields. Spores are usually air-borne and require a wet surface for germination. The Sacramento Valley, with its rivers, irrigation canals, and rice fields is noticeably more humid in summer than the San Joaquin Valley. The average relative humidity at 10 pm during August in Sacramento has been measured at 62%, compared with 42% at Fresno, in the San Joaquin Valley (12). With higher humidity, dew is also more common in the Sacramento Valley. Safflower frequently follows rice, so safflower fields are frequently adjacent to rice fields. From those observations it can be hypothesized that seed fungal infection would be higher in the Sacramento Valley. The wide range of FFA values found in grower samples from the Sacramento Valley certainly supports that hypothesis.

The *Alternaria* sp. isolated from seeds in this study should not be confused with the safflower leaf spot pathogen, *Alternaria carthami*. The fungus in this study is not an aggressive pathogen on green safflower plants. In the field it usually invades only senescent tissue. The receptacle of the infected head is dark in color, and the bristles thereon are easily detached. Seeds from such heads are often discolored, although a lack of discoloration does not mean that the seeds are free of infection. The *Alternaria* sp. can invade the safflower seed and cause degradation of safflower oil. The degree of degradation depends on the ramification of the fungus throughout the seed. In the greenhouse tests, oil content and yield were probably depressed by the fungus depriving the filling seeds of substrate. Since conditions for infection are not likely to be as favorable in the field as in the greenhouse, it is believed that in the field this *Alternaria* would cause only high FFA as it spread from senescent plant tissue onto the seeds in late stages of seed development.

The high levels of FFA in seeds from plants grown in Sacramento Valley fields and in the greenhouse, and in oil from in vitro studies, were in all probability caused by the action of lipases.

ACKNOWLEDGMENTS

This study was supported by a research grant from Pacific Vegetable Oil International, Inc. B.L. Brannaman gave chromatograhic assistance. D.H. Hall and J.M. Klisiewicz, Department of Plant Pathology, University of California, Davis, provided advice.

REFERENCES

1. Brockerhoff, H., and R.G. Jensen, "Lipolytic Enzymes," Academic Press, Inc., New York, NY, 1975.
2. Heaton, T.C., M.S. Thesis, 1977, University of California, Davis, CA.
3. Hansen, A.P., R.E. Welty, and R. Shen, J. Agric. Food Chem. 21:665 (1973).
4. Hoover, R., S.F. Laurentius, and K.G. Gunetileke, JAOCS 50:65 (1973).
5. Bennett, G.A., S. Freer, and D.L. Shotwell, Ibid. 53:53 (1976).
6. Ashworth, L.J., J.L. McMeans, B.R. Houston, M.E. Whitten, and C.M. Brown, Ibid. 48:129 (1971).
7. Kurnick, E., Proceedings of the Fourth International Sunflower Conference, 1970, pp. 149-161.
8. Zimmer, D.E., and D.C. Zimmerman, Crop Sci. 12:859 (1972).
9. Frey, K.J., and E.G. Hammond, JAOCS 52:358 (1975).
10. Brannaman, B.L., and J.E. Ruckman, unpublished procedure, Dept. of Agronomy and Range Science, University of California, Davis.
11. Hinz, P.N., and H.A. Eagles, Crop Sci. 16:280 (1976).
12. Climatological Data, California, U.S. Dept. of Commerce in cooperation with Dept. of Water Resources, State of California, 1970-1976.

[Received September 6, 1977]