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EFFECT OF PHENOLIC COMPOUNDS IN *LYCOPERSICON ESCULEUTUM* ON THE SYNTHESIS OF ETHYLENE

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Key Word Index—*Lycopersicon esculeutum*; Solanaceae; tomato; ethylene synthesis; naringenin; cinnamic acids; climacteric; indolyl-3-acetic acid.

Abstract—Caffeic, coumaric, sinapic and ferulic acids and naringenin were found in green tomato fruit. Chlorogenic acid accounted for 75% of the total phenolics in mature green fruit but only 35% in ripe fruit. There was very little change in the phenolic composition of the flesh of the fruit during ripening, whereas in the skin, naringenin increased markedly at the onset of the climacteric and three unidentified compounds increased during the climacteric rise. The increase in the concentration of naringenin was accompanied by an increase in the production of ethylene in the skin. Investigation of three systems producing ethylene from 4-methylmercapto-2-oxobutyric acid in the presence of peroxidase, showed that only *p*-coumaric acid or naringenin were capable of acting as phenolic substrates, the other phenolic compounds being inhibitory.

INTRODUCTION

PREVIOUS work with floret tissue of cauliflowers[1] and with tomato tissue[2] has implicated a peroxidase, a phenolic compound, and either peroxide or hydroperoxide, in the enzymic conversion of 4-methylmercapto-2-oxobutyric acid to ethylene. It has also been shown[3] that indolyl-3-acetic acid (IAA) is oxidized and ethylene produced from 4-methylmercapto-2-oxobutyric acid in the presence of peroxidase and a suitable phenolic compound. Work on the synthesis of ethylene in cauliflower extracts[1] demonstrated that dihydroxyphenols extended the lag phase of ethylene production. Yang[4] also reported that ethylene formation from methional by peroxidase was stimulated by monohydric phenols and inhibited by *O*-dihydric phenols. Observations that the oxidation of IAA by horseradish peroxidase[5] and by an enzyme from peas[6] was stimulated by monophenols and inhibited by di- and poly-phenols have been recorded and IAA oxidase in pineapple tissue[7] has been shown to be stimulated by *p*-coumaric acid and inhibited by ferulic acid, both phenols occurring in the tissue as esters. The involvement of phenolic compounds in ethylene biosynthesis thus seems to be well established.

On the other hand, the identification of phenolic compounds in tomatoes is not complete and little work has been attempted on quantitative changes during the climacteric rise. It therefore appeared of interest to identify and compare the concentrations of phenolic compounds in the mature green fruit, to follow any changes that might occur during the ripening process and to investigate whether these phenolic compounds stimulated or inhibited the production of ethylene in the *in vitro* system.

[1] MAPSON, L. W. and WARDALE, D. A. (1968) *Biochem. J.* **107,** 433.
[2] MAPSON, L. W. and WARDALE, D. A. (1971) *Phytochemistry* **10,** 29.
[3] MAPSON, L. W. and WARDALE, D. A. (1972) *Phytochem.* **11,** 1371.
[4] YANG, S. F. (1967) *Arch. Biochem. Biophys.* **122,** 481 (1967).
[5] KENTON, R. H. (1955) *Biochem. J.* **59,** 110.
[6] GOLDACRE, P. L., GALSTON, A. W. and WEINTRAUB, R. L. (1953) *Arch. Biochem. Biophys.* **43,** 358.
[7] GORTNER, W. A., KENT, M. J. and SUTHERLAND, G. K. (1958) *Nature* **181,** 630.

RESULTS

Unhydrolysed Extract—Total Phenols

A slight fall in total phenols was observed between the immature green tomato and the pre-climacteric fruit and this was followed by a sharp increase as the fruit ripened. This pattern was observed for the two seasons 1971 and 1972, although the amount of total phenols in 1972 was always higher.

Chlorogenic Acid

The amount of chlorogenic acid present in a mature green fruit varied during a season between 160 and 240 μg/10 g. This is in agreement with a figure of 180 μg/10 g reported by Jurics.[8] Chlorogenic acid accounted for a very high proportion of the total phenols found in mature green fruit varying from 50 to 75%. This estimation differs from the results of Walker,[9] whose semi-quantitative method was based on the size and intensity of the fluorescent spots on the chromatogram.

TABLE 1. CONCENTRATION OF CHLOROGENIC AND CAFFEIC ACIDS IN TOMATO FRUITS AT VARIOUS STAGES OF MATURITY

Compound	Immature green		Preclimacteric		Onset of climacteric		Postclimacteric	
	μg/10 g	Total phenols (%)	μg/10 g	Total (%)	μg/10 g	Total (%)	μg/10 g	Total (%)
Chlorogenic acid 1971 expt.	310	61	240	62	210	46	270	30
Chlorogenic acid 1972 expt.	410	73	238	75	218	40	381	35
Caffeic acid* 1972 expt.	182	72	141	75	132	62	232	53

* Determined in hydrolysed extracts.

The amount of chlorogenic acid present in a tomato at various stages of maturity, in two separate seasons, is shown in Table 1. There is a fall in the concentration of the acid as the green fruit matures, followed by an increase during the climacteric. This increase, however, is not reflected as a percentage of total phenols for the percentage of chlorogenic acid falls markedly after the mature green state due to a sharp increase in other phenolic compounds. Determination of the amount of chlorogenic acid in various parts of a mature green tomato (Table 2) showed that the largest concentration of both total phenols and chlorogenic acid was present in the parenchymatous tissue and seeds.

Identification of Remaining Phenolic Compounds in Green Fruit

Although other workers[9] have reported that ferulic, *p*-coumaric and caffeic acids are present as free acids in the tomato fruit walls, no such acids could be identified in the unhydrolysed fraction after chromatography. In view of this and the fact that phenolics in

[8] JURICS, E. W., (1966) *Z. Levensmittelunters. Forsch.* **132,** 193.
[9] WALKER, J. R. L. (1962) *J. Sci. Food Agric.* **13,** 363.

TABLE 2. CONCENTRATION OF TOTAL PHENOLS AND CHLOROGENIC ACID IN DIFFERENT PARTS OF THE MATURE GREEN FRUIT

	Outer wall of the pericarp	Inner and radial wall of the pericarp	Parenchymatous tissue and seeds
Wt. of fruit (gm)	35·5	11·7	9·5
Total phenols (μg/10 g)	140	257	990
Chlorogenic acid (μg/10 g)	70	193	740
Chlorogenic acid (% of total phenols)	50	75	75

fruits generally occur as quinic acid esters or glycosides,[10] half the aqueous extract was hydrolysed. Total phenols in the hydrolysate showed a similar pattern of change to the unhydrolysed total phenols. Ferulic, sinapic, caffeic, *p*-coumaric acids and naringenin were identified. An unidentified substance different from quercetin remained at the origin in toluene–HOAc–H_2O (10:7:3, upper), had an R_f of 0·56 in *n*-BuOH–HOAc–H_2O (4:1:5, upper), gave a positive reaction with $FeCl_3$–$K_3Fe(CN)_6$ and was pale yellow in UV light changing to light brown with ammonia.

Changes in the Hydrolysed Phenolic Compounds During Ripening

The concentrations of caffeic acid in hydrolysed extracts from tomatoes at various stages of maturity are shown in Table 1. The high percentage of caffeic acid of the total phenols present in both the immature and mature green fruit was in agreement with the percentage of chlorogenic acid found in the unhydrolysed extracts, but more caffeic acid was found as the fruit ripened than can be accounted for from hydrolysis of chlorogenic acid alone and suggested the presence of another caffeic acid derivative. Previous workers[11] have reported two caffeic acid derivatives present in canned tomato pastes. In the mature green fruit the other phenolics were present in only small quantities and from a typical experiment where caffeic acid accounted for 80% of the hydrolysed phenols, the remaining phenolics were: 5·5% ferulic acid, 4·5% sinapic acid, 3·5% coumaric acid, 2% naringenin and 4·5% unidentified compound. As the fruit ripens, ferulic, sinapic and *p*-coumaric acids show only a small increase in concentration, whereas marked changes occur both with naringenin and the unidentified compound, with the former increasing from 4 μg/10 g tissue in the green fruit to 84 μg in the ripe and the latter from 21 to 132 μg. It should also be noted that during the period during which the climacteric rise started, only naringenin had increased sharply, the unknown phenolics increasing during the ripening phase. In the mature green fruit only one unidentified compound remained on the base line in the TAW solvent whereas in the ripe fruit two additional compounds were present. This was shown by eluting the origin after TAW development and re-chromatography in BAW (4:1:55, upper) as solvent, when the following R_f's were noted: 0·69 and 0·88 and the original compound with an R_f of 0·57. Both the unknowns from ripe fruit were yellow in UV light and light brown in the presence of ammonia. An authentic sample of quercetin (R_f 0·78) did not correspond to either of the unknowns.

[10] HARBORNE, J. B. (1964) in *Biochemistry of Phenolic Compounds* (HARBORNE, J. B., ed.), pp. 129, Academic Press, London.

[11] RIVAS, N. and LUH, B. S. (1968) *J. Food Sci.* **33**, 358.

In view of the report[12] that naringenin was present in the skin of tomato fruits, it was decided to check whether the large increase in naringenin during ripening was confined to the skin. The amount of total phenols present in the skin and the peeled fruit were markedly different. The flesh phenolics were 460 μg/10 g in the mature green and 390 μg/10 g for the ripe fruit, a fall in phenolics during ripening which was not in agreement with the figures for the whole fruit. However, the skin phenolics increased from 710 μg/10 g in the green skin to 2280 μg in the ripe state, and it would appear that the changes in the concentration of phenolic compounds during ripening could well be taking place solely in the skin tissue.

The concentration and percentage of caffeic acid, naringenin, the unidentified compounds and ferulic, sinapic and coumaric acids, eluted together, from both skin and peeled fruit and the changes that occur as the fruit ripens are shown in Table 3.

TABLE 3. CONCENTRATION OF HYDROLYSED PHENOLIC COMPOUNDS IN EXTRACTS FROM THE PEELED FRUIT AND THE SKIN OF TOMATOES, AT VARIOUS STAGES OF MATURITY

	Preclimacteric		Onset of climacteric		Postclimacteric	
	μg/10 g	%	μg/10 g	%	μg/10 g	%
			Peeled fruit			
Total phenols	490	—	510		390	—
Unidentified compounds	17	4	19	3	48	13
Caffeic acid	378	78	421	83	290	74
Naringenin	12	3	13	3	8	2
r-Coumaric, Sinapic, Ferulic	75	15	56	11	44	11

	Preclimacteric		Onset of climacteric		Postclimacteric		Overripe	
	μg/10 g	%	μg/10 g	%	μg/10 g	%	μg/10 g	%
				Skin tissue				
Total phenols	710	—	770	—	2280	—	2200	—
Unidentified compounds	55	8	85	10	610	31	690	33
Caffeic acid	610	85	555	72	580	29	645	30
Naringenin	11	2	73	9	640	32	508	24
p-Coumaric, Sinapic, Ferulic	40	5	69	9	152	8	257	12

In the peeled fruit little change occurred during ripening with the percentage of caffeic acid remaining high. Naringenin was present in the flesh in a small quantity but did not increase during ripening. The other phenolic acids also showed little significant change but *p*-coumaric acid (17·5 μg/10 g tissue) was identified in the ripe peeled fruit.

In the skin tissue, however, the percentage of caffeic acid fell during the ripening phase whilst naringenin and the unidentified phenolic compounds, confirmed in ripe skin as composed of three compounds, rose. There was also an increase in concentration, although

[12] WU, M. and BURRELL, R. C. (1958) *Arch. Biochem. Biophys.* **74**, 114.

much smaller, in the combined fraction containing ferulic, sinapic and *p*-coumaric acids and, although these were not estimated individually, the evidence from the whole fruit indicated that all three increased by about the same amount.

The only discrepancy between duplicate experiments occurred with the skin from fruit at the onset of the climacteric, where the naringenin concentration varied a great deal. The reason for this could be in the sampling as the skin was composed of a mixture of green and pink areas. Accordingly, the skin from a batch of partially ripe fruit was divided into two parts, the green and the slightly coloured. The amount of caffeic acid from the hydrolysed extracts were 358 μg (65% of total phenols) from the green areas and 430 μg (39%) from the coloured areas and for naringenin, 71 μg (13%) and 360 μg (33%). This latter figure, when compared with the amount of naringenin found in the ripe and over-ripe skin suggested that the flavanone was formed during the early stages of ripening and did not increase during the post-climacteric period. Naringenin occurs in the free state and there was no evidence for the occurrence of a glycoside in the fruit.

Effect of Tomato Phenolic Compounds on the Production of Ethylene

Three systems have been shown to stimulate the production of ethylene from 4-methylmercapto-2-oxobutyric acid:[3] (a) indolyl-3-acetic acid in the presence of peroxidase, a phenolic acid and a sulphinic acid; (b) peroxidase, a phenolic acid and a sulphinic acid in the presence of systems generating hydroperoxide, such as lipoxygenase and linolenic acid; and (c) the indolyl-3-acetic acid system with the addition of a hydroperoxide. Of these systems only the second has been shown to be present in tomato tissue.[2]

Chlorogenic acid, caffeic acid, ferulic acid and sinapic acid at 0·05 and 0·5 mM concentrations were all incapable of acting as substrates in any of the three systems tested. Only naringenin and *p*-coumaric acid stimulated the production of ethylene from the oxo acid in the presence of IAA, peroxidase and a sulphinic acid (Table 4, system *A*) and in the system where lipoxygenase and linolenate were also present (system *C*). Naringenin, but not *p*-coumaric acid, promoted the production of ethylene from the oxo acid when peroxidase, sulphinic acid and hydroperoxide were present (system *B*), but ethylene was also produced from this system when the methyl ester of *p*-coumaric replaced the acid. This is in agreement with earlier work,[13] where the methyl ester of *p*-coumaric acid promoted ethylene synthesis more vigorously than the acid. In all three systems there was no production of ethylene in the absence of a phenolic compound. It was also shown that when chlorogenic acid and naringenin were added together in equal concentrations the production of ethylene was completely inhibited. In view of these experiments it was not surprising that an extract of unhydrolysed phenolics from ripe tomato skin, which contained a high proportion of chlorogenic acid, also inhibited the production of ethylene. Naringenin, however, isolated from a hydrolysed extract of ripe tomato skin, was stimulatory (Table 4).

Although it has been shown that two phenolic compounds present in tomato extracts are capable of acting as substrates in the production of ethylene, this provides no proof that they are involved *in vivo*. A comparison was therefore made between the production of ethylene from whole fruit, peeled fruit and skin and the concentration of naringenin present, at various stages of maturity. The results, Table 5, showed that the increase in ethylene production during the climacteric rise in whole fruit is accompanied by an increase

[13] MAPSON, L. W. and MEAD, A. (1968) *Biochem. J.* **108,** 875.

of naringenin and that this increase in ethylene production is confined mainly to the skin where the naringenin is concentrated.

TABLE 4. THE EFFECT OF VARIOUS PHENOLIC COMPOUNDS ON THE PRODUCTION OF ETHYLENE FROM 4-METHYL-MERCAPTO-2-OXOBUTYRIC ACID

System	Additions	Ethylene produced (μl) min 10	20	30
A	*p*-Coumaric acid (0·05 μmol)	1·0	1·18	1·24
	p-Coumaric methyl ester (0·05 μmol)	1·42	1·78	1·8
	Naringenin (0·5 μmol)	2·2	2·88	3·5
	Naringenin (0·5 μmol) + Chlorogenic (0·5 μmol)	0	0	0
B	Naringenin (0·5 μmol)	0·08	0·15	0·17
	p-Coumaric acid (0·05 μmol)	0	0	0
	p-Coumaric methyl ester (0·05 μmol)	0·12	0·16	0·22
C	*p*-Coumaric acid (0·05 μmol)	0·2	0·36	0·46
	Naringenin (0·5 μmol)	2·3	2·9	3·5
	Naringenin + Chlorogenic (0·5 μmol)	0	0	0
	Unhydrolysed phenolics	0	0	0
	Naringenin from tomato	1·2	1·76	1·76

Flasks contained System *A*. Benzene sulphinic acid (1 μmol), oxo acid (10 μmol), IAA (1 μmol) and peroxidase (1·5 units) in 10 ml 0·1 M sodium phosphate buffer, pH 5·5, containing EDTA (20 μmol). *B*. Benzene sulphinic acid (5 μmol), oxo acid (10 μmol), peroxidase (1·5 units), linolenate (10 μmol) and lipoxygenase (4000 units) in 10 ml sodium phosphate buffer, pH 6·0. *C*. Addition of the two systems with benzene sulphinic acid (1 μmol), IAA (0·5 μmol) and a pH of 5·5. The unhydrolysed phenolics ≡ 1 g. fr. wt tissue were prepared from ripe tomato skin and the naringenin ≡ 2 g tissue was isolated from a hydrolysed extract from ripe skin.

Tissue Plugs

No significant stimulation or inhibition occurred when naringenin, *p*-coumaric acid, ferulic acid or chlorogenic acid at a concentration of 0·1 mM were added to tissue plugs from fruit in the pre-climacteric or onset of climacteric phases. The only inhibition noted, 35% in 5 hr and 40% in 20 hr, was from an unhydrolysed extract from ripe skin (1 g fr. wt tissue) added to the solution surrounding green tissue plugs.

DISCUSSION

Derivatives of four hydroxycinnamic acids and naringenin have been shown to be present in green and ripe tomato extracts. This is the first report of a sinapic derivative being present in tomatoes, but it is commonly found in fruits when derivatives of the other three phenolic acids occur.[14]

Only two of the phenolic compounds present in tomato fruits are capable of acting as substrates in the formation of ethylene from the three model systems investigated. The amount of naringenin and *p*-coumaric present in the mature green fruit is in excess of that required to account for the observed production of ethylene from peeled tissue plugs or the skin tissue. During the climacteric, ethylene increases mainly in the skin, and it is in this

[14] VAN BUREN, J. (1970) in *The Biochemistry of Fruits and Their Products* (HULME, A. C., ed.), **1**, pp. 271, Academic Press, London.

same tissue that we have observed the marked increase in naringenin. If these phenolics are involved in ethylene production *in vivo*, then the large amount of chlorogenic acid, which is present in fruits at all stages of maturity and which would be a potent inhibitor of ethylene production, must be in a separate compartment from the stimulating phenolics.

TABLE 5. RELATIONSHIP BETWEEN THE AMOUNT OF ETHYLENE PRODUCED BY WHOLE FRUIT, PEELED TOMATO PLUGS OR SKIN TISSUE AND THE CONCENTRATION OF NARINGENIN

	Condition of fruit	Ethylene produced μl/10 g/hr	Naringenin content μg/10 g
Whole fruit	Preclimacteric	0.004	10
	Onset of climacteric	0·025	29
	Postclimacteric	0·23	84
Peeled tissue plugs	Preclimacteric	0·18	12
	Onset of climacteric	0·38	13
	Postclimacteric	0·31	8
Skin tissue	Preclimacteric	0·13	11
	Onset of climacteric	0·94	73
	Postclimacteric	1·84	640

Ethylene production was measured from 12 tomato peeled plugs (10 g) and skin tissues (4 g) immersed in 10 ml 0·2 M sodium phosphate buffer, pH 5·5, containing mannitol (0·2 m) and EDTA (2 mM) over a 5 hr period in oxygen at 25°. The flasks were evacuated at the start to remove any residual ethylene.

One should also consider the possible role of these tomato phenolics in the enzymic degradation of the plant growth hormone indolyl-3-acetic acid. Previous workers[15] stated that the activity of this enzyme in tissue may be regulated by the relative concentrations of phenolic co-factors and inhibitors and this has been shown in peas[6] and pineapples.[7] More recently, Frenkel[16] proposed that growth regulatory systems in fruits undergo changes as a necessary prerequisite for ripening and that this could account for the changes in the resistance to ripening and to ethylene action. He further stated that a capacity for IAA oxidation is developed in fruits during ripening, irrespective of any changes in enzyme activity. In this present work we have observed an increase in naringenin at the onset of the climacteric and this would lead not only to an increase in the ethylene produced by the fruit but also to an increase in the oxidation of indolyl-3-acetic acid. This destruction of the hormone would increase in the skin of the tomato during the ripening phase.

EXPERIMENTAL

Enzymes and chemicals. Soybean lipoxygenase (134 000 Sigma units/mg), horseradish peroxidase (300 purpurogallin units/mg) and indolyl-3-acetic acid were obtained commercially. The oxo acid, 4 methyl-mercapto-2-oxobutyric acid, was prepared as described previously.[17] Linolenic acid was used as the ammonium salt.

Tomatoes. Tomato plants (*Lycopersicum esculentum* Mill, variety Eurocross BB) were grown in the glasshouse and the fruit was sampled according to its appearance. Preclimacteric fruit denotes mature green fruit; onset of climacteric, fruit with a trace of colour; climacteric, half ripe fruit and post-climacteric, firm red

[15] GALSTON, A. W., LAVEE, S. and SIEGAL, B. Z. (1968) in *Biochemistry and Physiology of Plant Growth Substances* (WIGHTMAN, F. and SETTERFIELD, G., eds.). p. 455, Runge Press, Ottawa, Canada.
[16] FRENKEL, C., (1972) *Plant Physiol.* **49**, 757.
[17] MAPSON, L. W., MARCH, J. F. and WARDALE, D. A. (1969) *Biochem. J.* **115**, 653.

fruit. Plugs of tissue 10 mm dia. were cut from the flesh of the tomato walls with the skin attached to one face of the plug. 12 disks (6 g) were immersed in 10 ml 0·2 M sodium phosphate buffer, pH 5·5, containing mannitol (0·2 M) and EDTA (2 mM) in a 50 ml flask with oxygen, shaken at 25° and gas samples removed at intervals for ethylene determinations.[18]

Extraction of the phenolics. Tissue from 400 g of tomatoes was cut into 600 ml of boiling MeOH, left boiling for 5 min and then disintegrated in a blender. The suspension was filtered through muslin and the residual pulp re-extracted with 80% MeOH. The extracts were combined, clarified by high speed centrifuging and concentrated *in vacuo*. Half the aqueous concentrate was adjusted to pH 3·2 with dil. HCl and extracted 2× with light petrol. to remove the carotenoids and then with EtOAc (×3). The EtOAc extracts were dried and concentrated.

Hydrolysis of the phenolics. This was carried out by a modified method of El Basyouni *et al.*[19] Half the aqueous concentrate was held in an ice bath under N_2. Strong NaOH (O_2 free) was then added to give a final concentration equivalent to 2 N. The tube was stoppered and kept at +1° overnight. The contents were acidified with conc. HCl to pH 2·8, extracted 2× with light petrol. and then the phenolic acids extracted into Et_2O (×3). The dried extract was concentrated.

Separation and purification of compounds. The identity of chlorogenic acid from the unhydrolysed extract and the other phenolic compounds present in the hydrolysed extract were confirmed by R_f in 2 solvents, *n*-BuOH–HOAc–H_2O (4:1:5, upper) and toluene–HOAc–H_2O (10:7:3, upper) on PC, their appearance in UV light, their specific colour reactions with *O*-dianisidine diazonium salt, diazotized *p*-nitroaniline[20] and Hoepfner reagent[9] and their UV spectra after elution from the chromatograms.

The Folin-Denis colorimetric method, modified by Swain and Hillis[21] and the UV spectra of purified compounds eluted from PC with 3 ml 0·1 N NaOH were used for the quantitative estimation of the phenolic compounds. The unknown compounds were estimated as caffeic acid.

The flavanone present in the unhydrolysed extract had the same R_f as an authentic sample of naringenin in three solvents BAW, 0·94, TAW, 0·11 and 2% AcOH, 0·2.

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[18] MAPSON, L. W., and WARDALE, D. A. (1967) *Biochem. J.* **102,** 574.
[19] EL-BASYOUNI, S. Z., NEISH, A. C. and TOWERS, G. H. N. (1964) *Phytochemistry* **3,** 627.
[20] SWAIN, T. (1953) *Biochem. J.* **53,** 200.
[21] SWAIN, T. and HILLIS, W. E. (1959) *J. Sci. Food Agric.* **10,** 63.