Phytochemistry, Vol. 20, No. 7, pp. 1473–1475, 1981.
Printed in Great Britain.

0031-9422/81/071473-03 $02.00/0
© 1981 Pergamon Press Ltd.

ROLE OF IAA-OXIDASE IN THE FORMATION OF ETHYLENE FROM 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID

A. VIOQUE, M. A. ALBI and B. VIOQUE

Instituto de la Grasa y sus derivados, Av. P. G. Tejero, 4 Sevilla, Spain

(*Revised received* 20 *November* 1980)

Key Word Index—*Olea europea*; Oleaceae; IAA-oxidase; 1-aminocyclopropane-1-carboxylic acid; ethylene; pyridoxal phosphate.

Abstract—The IAA-oxidase system of olive tree (*Olea europea*) in the presence of its substrate, IAA, and cofactors, DCP and Mn^{2+}, forms ethylene from 1-aminocyclopropane-1-carboxylic acid (ACC) bound as a Schiffs base to pyridoxal phosphate. Similarly, olive leaf discs upon incubation with ACC liberate considerable amounts of ethylene. The results suggest that this IAA-oxidase system may be the one active in the last step in the biosynthesis of ethylene from methionine.

INTRODUCTION

Investigations of the biosynthesis of ethylene from methionine in vegetable tissues culminated with the discovery [1, 2] that 1-aminocyclopropane-1-carboxylic acid (ACC) is its immediate precursor, and led to the following pathway: methionine (Met) → *S*-adenosyl-methionine (SAM) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene.

In previous studies [3, 4] on the abscission of olive fruit, we described the characterization of the IAA-oxidase system from different tissues of the olive tree. The outstanding role played by this system in ripening and senescence is widely recognized; however, its precise function is controversial, as it shows, even at the isoenzyme level, two enzymic activities, IAA-oxidase and peroxidase [5–8].

The work now reported shows that our IAA-oxidase [10] on the formation of ethylene from α-keto-γ-methylthiobutyric acid (KMB), the more recent one by Konze and Kende [11] using ACC, as well as the ideas of Adams and Yang [1] on the participation of hydrogen peroxide in the breaking of the cyclopropane ring prior to the ethylene formation, suggest that the IAA-oxidase/peroxidase system from olive tree could also be active '*in vivo*' in the ACC → ethylene step; this system has all the properties associated with this activity.

The work now reported shows that our IAA-oxidase system, in the presence of its substrate (IAA) and the proper cofactors, acts '*in vitro*' on a pyridoxal phosphate Schiffs base of ACC, forming ethylene.

RESULTS

Ethylene formation upon incubation of ACC with IAA-oxidase

The IAA-oxidase system from olive tree leaves when incubated with ACC (1–10 mM), under the conditions found [4] to be optimal to develop IAA-oxidase activity, produced small amounts of ethylene (Table 1, experiment 2). However, upon addition of pyridoxal phosphate, the amount of ethylene evolved was twenty times as much (Table 1, experiment 1). Experiments at different pHs showed that the evolution of ethylene was maximum at pH 5.7; it has been found in previous work [4] that the IAA-oxidase activity of the extract also has a maximum at the same pH.

The formation of detectable amounts of ethylene in the absence of enzymic extract or IAA (Table 1, experiments 4 and 5) was at first surprising. However, because Mn^{2+} is present in the incubation mixture, a likely explanation could be the formation of a complex amino acid–metal–pyridoxal phosphate similar to that described by Metzler *et al.* [12]; this complex could generate ethylene chemically. In accordance with this view, no ethylene was detected (Table 1, experiments 6 and 7) when Mn^{2+} was omitted.

Table 1. Ethylene formation upon incubation of ACC with IAA-oxidase of olive tree

Expt. No.	Reaction mixture	Ethylene (nmol/mg protein)
1	Complete	845.8
2	No pyridoxal phosphate	42.4
3	No ACC	0
4	No enzyme	24.5
5	No IAA	18.5
6	No enzyme, no Mn^{2+}	0
7	No IAA, no Mn^{2+}	0
8	No DCP	9.8
9	No Mn^{2+}	4.2
10	Atm. N_2	7.6
11	Boiled enzyme	36.7

Reaction mixture: ACC (10 mM), enzyme extract (96.7 μg protein), pyridoxal phosphate (0.4 mM), IAA (0.2 mM), DCP (0.3 mM), Mn^{2+} (0.3 mM), phosphate buffer (0.02 M, pH 5.7), final vol. 5 ml, 30°, darkness, 24 hr.

When any of the typical cofactors (dichlorophenol, DCP or Mn^{2+}) of the IAA-oxidase activity was omitted, the formation of ethylene was practically zero (Table 1, experiments 8 and 9). Assays under N_2 or using boiled extracts (Table 1, experiments 10 and 11) showed once again that the process is aerobic and involves a heat-labile enzymic system.

As the IAA-oxidase activity shows inhibition by excess substrate [4], the effect of increasing concentrations (0.025 to 1.20 mM) of IAA on the volume of ethylene evolved was studied; the results showed that ethylene evolution increased with increasing concentrations of IAA until a maximum was reached at 0.40 mM concentration of substrate, and then decreased. Table 2 shows the effects of several typical inhibitors of the IAA-oxidase system.

Ethylene formation upon incubation of ACC with olive tree tissues

Table 3 shows how olive tree leaf discs, incubated with 1 mM ACC, form up to 160 times more ethylene than water alone (endogenous ACC). Peduncle sections and olive discs (from small, immature fruits) form 73.5 and 6.6 nmol/g (24 hr), respectively.

The effects of anaerobic conditions and of the immersion of the olive tree leaf discs in boiling water on ethylene evolution are also shown in Table 3. The results confirm the requirement of oxygen in the ACC → ethylene step and the involvement of a heat-labile enzymic system.

Similarly as found for other vegetative tissues [2], olive tree leaf discs ground with water or in an appropriate buffer lost their ability to form ethylene from ACC. Lürssen *et al.* [2] regarded this as proof that intact, organized tissue is required for enzymic activity. Our results with purified leaf extracts are in disagreement with this and we consider instead that the polyphenols released following the cell disruption inhibit the IAA-oxidase activity and, consequently, the formation of ethylene. The inhibition of the IAA-oxidase by polyphenols has been previously demonstrated [4]. The same explanation was set forth by Ku *et al.* [9] in their studies of ethylene formation from KMB in crude tomato extracts. Table 4 summarizes the effects '*in situ*' of the same inhibitors assayed with purified extracts.

Table 2. Inhibition of ethylene formation by action of IAA-oxidase on ACC

Inhibitor (10 μM)	% Inhibition
Co^{2+}	47
CN^-	51
NaN_3	9
EDTA	36
Catalase (5 μg)	55

Reaction mixture: as in Table 1 plus the inhibitors (10 μM) shown above.

Table 3. Ethylene formation upon incubation of ACC with olive tree leaf discs

Incubation solution	Ethylene (nmol/g/fr. wt)
ACC, 1 mM	773.7
H_2O (endog. ACC)	4.7
ACC, 1 mM, atm. N_2	0
ACC, 1 mM, discs, 2 min in boiling H_2O	0

Incubation of ACC (1 mM, 1 ml) with three discs of olive tree leaf (10 mm ϕ, 68 mg), 30°, darkness, 24 hr.

DISCUSSION

The above results provide evidence that the purified IAA-oxidase system from olive tree in the presence of its substrate (IAA) and cofactors (DCP and Mn^{2+}) is able to decompose the Schiffs base of ACC and pyridoxal phosphate, forming ethylene. The properties of this system, previously studied [4], are considered [1, 2, 11] to be characteristic of the enzyme(s) presumed to be active in the last step of ethylene biosynthesis (ACC → ethylene). Similarly, the parallelism observed between the inhibitors of both IAA-oxidation [4] and ethylene formation (Table 2) of the IAA-oxidase of olive tree, and the inhibitions found (Table 4) in the assays '*in situ*' indicate that this system might act also '*in vivo*' as the last step in ethylene biosynthesis.

The stimulating effect of pyridoxal phosphate on the rate of ethylene formation is significant. First of all, it can be taken as a proof of the need of its presence in the step SAM → ACC, as previously suggested [1, 2], to explain the inhibition due to aminoethoxyvinylglycine (AVG); also it supports the view [2] that pyridoxal phosphate remains bound as a Schiffs base to ACC until its degradation to ethylene has taken place. The well established fact [2, 11] that AVG does not inhibit the ACC → ethylene step may be interpreted to indicate that this inhibitor cannot act after formation of the Schiffs base.

In connection with the mechanism of the reaction ACC → ethylene, the inhibition exerted by catalase both '*in vitro*' and in the '*in situ*' assays with discs is interesting. This behaviour of catalase favours the hypothesis that hydrogen peroxide [1] or a peroxide radical [10, 14], previously formed by an oxidase, participates in the opening of the cyclopropane ring. This would also justify the dual (oxidase and peroxidase) function always exerted by the so-called IAA-oxidase system [4–8, 13].

The participation of the IAA-oxidase system and its substrate, IAA, in this step of the ethylene biosynthesis

Table 4. Inhibition of ethylene formation upon incubation of ACC with olive tree leaf discs

Inhibitor (10 μM)	% Inhibition
Co^{2+}	40
CN^-	28
N_3Na	42
EDTA	23
Catalase (5 μg)	45

Incubation of ACC (1 mM, 1 ml) with a disc of leaf (10 mm ϕ, 20 mg), 30°, darkness, and the inhibitors (10 μM) shown above.

would also explain the well known stimulation of ethylene production in vegetative tissues pre-treated with IAA. This IAA-induced ethylene production responds positively to increasing concentrations of IAA and then decreases. This behaviour is very close to the known [4, 6] inhibition of the IAA-oxidase system by excess substrate and to the inhibition of ethylene evolution from ACC at concentrations higher than 0.40 mM IAA now found. The above interpretation is at variance with that of Yu and Yang [15], who consider that IAA exerts its stimulating effect by inducing the synthesis of the enzyme involved in the conversion of SAM into ACC.

EXPERIMENTAL

Preparation of the enzymic extract from olive tree leaves. The preparation was carried out as previously described [4]. The enzymic activities of the extract were: IAA-oxidase = 1.06 μmole IAA/min/mg protein; peroxidase (guaiacol) = $344\Delta A_{420}$/min/mg protein.

Incubation of ACC with enzymic extract. 50 μl of 1 M or 100 mM ACC and 200 μl 10 mM pyridoxal phosphate were brought to a vol. of 1 ml with 0.02 M phosphate buffer (pH 5.7) in a 15 ml vial. The vial was tightly stoppered with rubber cap and shaken 10 min at 30°. A freshly prepared soln containing 1 ml 1 mM IAA, 0.5 ml 3 mM DCP, 0.5 ml 3 mM Mn^{2+} and 2 ml 0.02 M phosphate buffer (pH 5.7) was injected, followed by 10 μl of enzymic extract. Final vol. 5 ml. The vial was incubated at 30°, in the dark, and gas samples (1 ml) of the head space were collected periodically, or after 24 hr, for ethylene determination.

Incubation of ACC with olive tree leaf discs. Discs (10 mm φ) were incubated at 30°, in the dark with 1 ml 1 mM ACC in 15 ml vial, tightly closed. Samples (1 ml) were withdrawn periodically or after 24 hr to estimate ethylene.

Ethylene determination. GLC, FID, glass column 3 m × 3 mm i.d., Chromosorb 102, oven temp. 40°, carrier gas He.

Acknowledgements—We thank Dr. Lürssen, Bayer AG, Leverkusen, for supplying a valuable sample of ACC. One of the authors (B.V.) is indebted to the "Juan March Foundation" for a fellowship.

REFERENCES

1. Adams, D. O. and Yang, S. F. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 170.
2. Lürssen, K., Naumann, K. and Schröder, R. (1979) *Z. Pflanzenphysiol.* **92**, 285.
3. Vioque, A. and Albi, M. A. (1975) *Grasas Aceites (Seville)* **26**, 73.
4. Vioque, A., Albi, M. A. and Vioque, B. (1978) *Grasas Aceites (Seville)* **29**, 391 and 397.
5. Sequeira, L. and Mineo, I. (1966) *Plant Physiol.* **41**, 1200.
6. Frenkel, Ch. (1972) *Plant Physiol.* **49**, 757.
7. Hoyle, M. C. (1977) *Plant Physiol.* **60**, 787.
8. Zmrhal, Z. and Machacková, I. (1978) *Phytochemistry* **17**, 1517.
9. Ku, H. S., Yang, S. F. and Pratt, H. K. (1969) *Phytochemistry* **8**, 567.
10. Mapson, L. W. and Wardale, D. A. (1972) *Phytochemistry* **11**, 1371.
11. Konze, J. R. and Kende, H. (1979) *Planta* **146**, 293.
12. Metzler, D. E., Ikawa, M. and Snell, E. E. (1954) *J. Am. Chem. Soc.* **76**, 648.
13. Hoyle, M. C. (1972) *Plant Physiol.* **50**, 15.
14. Fox, L. R., Purves, W. K. and Nakada, H. I. (1965) *Biochemistry* **4**, 2754.
15. Yu, Y. B. and Yang, S. F. (1979) *Plant Physiol.* **64**, 1074.