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# METABOLIC FINGERPRINTING OF SALT-STRESSED TOMATOES

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**Summary**. Increased salinisation of agricultural land is having a significant impact on agriculture, decreasing crop productivity. The research presented is part of a collaborative EU INCO-DC project. Data will be presented on two objectives: firstly the selection and screening of tomato varieties for potential salt tolerance and secondly, studies on the effect of salinity on fruit yield and quality.

Results indicated that a variety, Edkawy, displayed properties of salt tolerance hence it was selected as a model for metabolomic fingerprinting studies. Preliminary data obtained using Pyrolysis Mass Spectrometry (PyMS) and analysed by Principal Component Analysis (PCA) enabled discrimination between fruit according to ripeness stage, fruit ripened on- and off-the-vine and fruit artificially ripened with ethylene. Tomatoes grown under conditions of high and low-salt concentrations were analysed using Fourier Transform InfraRed spectroscopy (FTIR) with the aim of identifying biochemical features linked to salinity in the environment. FTIR spectra of whole tissue extracts are not amenable to visual analysis so evolutionary computer modelling methods were applied which were capable of classifying samples on their spectral characteristics. Genetic Programming (GP) models proved to be successful in enabling a chemical interpretation of biochemical fingerprint differences to be proposed. The authors acknowledge the support of the Analytical Biotechnology and Machine Learning group (http://www.aber.ac.uk/biology/research/abml.html).

**Key words:** metabolic fingerprinting – salt stress – tomatoes

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## Introduction

Environmental stresses such as salinity significantly affect crop growth and productivity (Adams et al., 1992). The problem is most severe in arid and semi-arid regions of the world where the use of irrigation practices is extensive and necessary to overcome the problems of drought, intermittent rain and to extend the growing season (Toenniessen, 1984; Ellis and Mellor, 1995). Although plant responses to salinity are one of the most widely researched subjects in plant physiology the mechanisms that impart salt tolerance are still unresolved (Munns, 2002). The study of salinity stress incorporates research ranging from adaptations of plants to salinity to genetic screening and manipulation (Shannon, 1997).

The work presented in this paper was conducted as part of a EU INCO-DC project entitled "Optimising marginal resources in intensive horticultural production in Southern Turkey and Northern Egypt". Tomato was selected as a model crop for these studies due to its commercial importance as a horticultural cash crop in many areas where exposure to increased salinisation occurs. Increasing salinity levels negatively affect germination, plant growth and fruit yield, resulting in economic losses (for a review see Cuartero and Fernandez-Minoz, 1999). One of the preliminary objectives was the selection and screening of various tomato varieties for salt tolerance in relation to plant growth and development. The screening of tomato varieties was performed using classical plant growth analysis which involves the collection of a series of sequential measurements describing plant size and form (Hunt, 1982). Of the varieties studied, Edkawy, an Egyptian beefsteak tomato, was identified as being potentially salt-tolerant (Mahmoud et al., 1986a & b; Johnson et al., 1999) and was selected for further study on tolerance mechanisms especially in relation to fruit quality.

In recent years, increased emphasis has been placed on fruit quality rather than quantity by the consumer, the retailer and the grower. However, the reliable assessment of quality is inexact. There is concern that a single parameter cannot define quality and even combinations of parameters, such as specific chemical attributes, in defining quality is questionable. This led us to develop the post-genomic technology of metabolomics to address issues of quality including the effect of salinity. The metabolome is a generic term for the total biochemical composition of a cell or tissue sample at any given time (Oliver et al., 1998). More recently, metabolic analyses have been categorised into four different strategies (Fiehn, 2001). Metabolite target analysis has been used to study primary effects of alterations (including mutations) but still includes extensive clean-up procedures to improve signal to noise ratios. An alternative approach is to focus on selected biochemical pathways which is often referred to as metabolite profiling. In instances where a comprehensive metabolic analysis is required including the identification of unknown metabolites and where there may be unforeseen metabolic consequences of for example manipulated plants, then this falls under the strategy of metabolomics. However, there is still much of a technology platform to be built in

this context when applied to plant analysis. Methods need to allow high and comprehensive recovery and reproducibility while providing selectivity and sensitivity. The approach adopted here was that of metabolic fingerprinting where samples can be rapidly classified according to their origin or their biological relevance.

Two techniques, Pyrolysis mass spectrometry (PyMS) and Fourier transform infra-red spectroscopy (FT-IR) were used. Both are physico-chemical methods that measure predominantly the bond strengths of molecules and the vibrations of bonds within functional groups respectively. In PyMS samples are pyrolysed at 530 $^{o}$C and the pyrolysate bombarded with low energy electrons to produce molecular and fragment ions, a majority of which are positively charged. These ionised fragments are accelerated through a quadrupole mass filter and separated according to their mass to charge ratio. The signal is then detected and amplified. The resultant spectrum shows ion count against mass to charge ratio (*m/z*), with the *m/z* ranging from 51–200 containing 150 variables (Goodacre and Kell, 1996).

FT-IR uses the vibrational characteristics within molecules to obtain a "fingerprint" spectrum with features defined by the functional chemical groups within the sample. The precise frequencies of light absorbed are related to the energies specific to the vibrational modes of each chemical group. The spectra in these experiments span from 4000 to 600 $cm^{-1}$ wavenumbers with a resolution of approximately 4 $cm^{-1}$. Each sample is thus characterised by 882 variables, each of which indicates the level of absorbance at a particular frequency of infra-red light. Spectra from PyMS and FT-IR are large multivariate data sets which are virtually uninterpretable by visual analysis. Hence, the use of chemometric methods is necessary to reduce the dimensionality of the data to enable interpretation. Principal Components Analyis (PCA) and Genetic Programming (GP) were used in the research presented here. The technique of PCA is a clustering method which acts to reduce the dimensionality of multivariate data whilst preserving most of the variance within it (Goodacre et al., 2000). GP is an evolutionary programming technique in which populations of computer programmes compete against each other to produce solutions to a problem. GPs can produce readily-interpretable mathematical models which subsequently allow the interpretation of complex biological spectra (Koza, 1992).

## Materials and Methods

### Plant Growth and harvesting

Tomatoes were grown in an open hydroponic drip irrigation system (Johnson et al., 1999). Two independent systems were established enabling two treatments, a control where irrigation water contained nutrients only and a salt treatment containing nutrients and supplementary NaCl at a concentration of 0.4%. For the PyMS experiments

on fruit ripening, fruits were harvested at the breaker stage (Stage 3, OECD tomato colour gauge www.oecd.org/agr/code/cont-e.htm.). Fruit were placed in desiccators and ethylene was injected to give a final concentration of 200 $\mu l.l^{-1}$, a concentration known to induce fruit ripening. Fruit were analysed when fully ripe (OECD stage 9). Fruit were also left to ripen on-the-vine and again were harvested when fully ripe. For the FT-IR analysis, fruit were harvested when fully ripe from control and salt-treated plants from the 1st and 2nd trusses.

**Sample preparation**

Fruits were washed in distilled water to remove any soluble surface contaminants. The inner pericarp, locular jelly, seeds and placental tissue were removed and discarded. The mesocarp (flesh) was then crushed to a pulp, the fresh weight measured and Milli-Q water added in a ratio of 2:1 (w/v) to improve the homogeneity of sample loading. After mixing, 1ml aliquots were pipetted into Eppendorf tubes and snap frozen in liquid nitrogen prior to storage at –70 °C.

**Biochemical analyses**

PyMS was carried out using a Horizon Instrument PYMS-200X. Ten samples from each treatment were run; aliquots of 5 µl were dried at 60 °C prior to being loaded and Curie-point pyrolysis was at 530 °C for 3 s, with a temperature rise time of 0.5 s from 100 to 530 °C. Data were collected for each sample over the *m/z* ranging from 51–200, resulting in a spectrum of 150 *m/z* intensities per sample. These data were normalised as a percentage of the total ion count to remove effects of the sample size.

The samples were thawed at room temperature and mixed prior to loading into the drilled wells on an aluminium plate for analysis by FT-IR; 5 µl of sample were loaded per well. Ten machine replicates were loaded for each tomato fruit sample. The plate was then dried at 50 °C for 45 min prior to loading onto the motorised stage of a reflectance thin-layer chromatography (TLC) accessory attached to a Bruker IFS28 FT-IR spectrometer (Bruker Ltd.) equipped with a mercury-cadmium-telluride (MCT) detector cooled using liquid $N_2$ as detailed in (Goodacre, et al., 1998; Timmins, et al., 1998).

The diffuse reflectance absorbance FT-IR spectra were collected over a wavenumber range from 4000 $cm^{-1}$ to 600 $cm^{-1}$ under the control of an IBM-compatible personal computer using OPUS 2.1 software running under the IBM OS/2 Warp operating system at a resolution of approximately 3.85 $cm^{-1}$. The resultant spectrum for each sample contained 882 variables. Spectra were acquired at a rate of 20 $s^{-1}$. To improve signal-to-noise ratio, 256 spectra were co-added, added sequentially as each was collected.

*Data analysis.* Plant growth data were analysed using Analysis of Variance (ANOVA) in Excel (Microsoft Office 97). The spectral data were analysed using MATLAB version 6.1.

## Results and Discussion

Preliminary PyMS analysis from mature green and fully ripe fruit provided data that showed clear evidence of clustering using PCA (Fig. 1). Although encouraging, this result was perhaps not surprising given the difference in the developmental stage of the fruit. Figures 2 and 3 show the PCA scores of data from fruits ripened either on- or off-the-vine or artificially ripened with ethylene, respectively. Clearly the data from fruits ripened off-the-vine clustered differently from those ripened on-the-vine and those from fruit ripened with ethylene treatment. Visually, these fruits were all classed at the same stage (OECD Stage 9) and hence all fruits analysed could be classified as fully ripe fruit. However, PCA analysis of the metabolic fingerprints showed

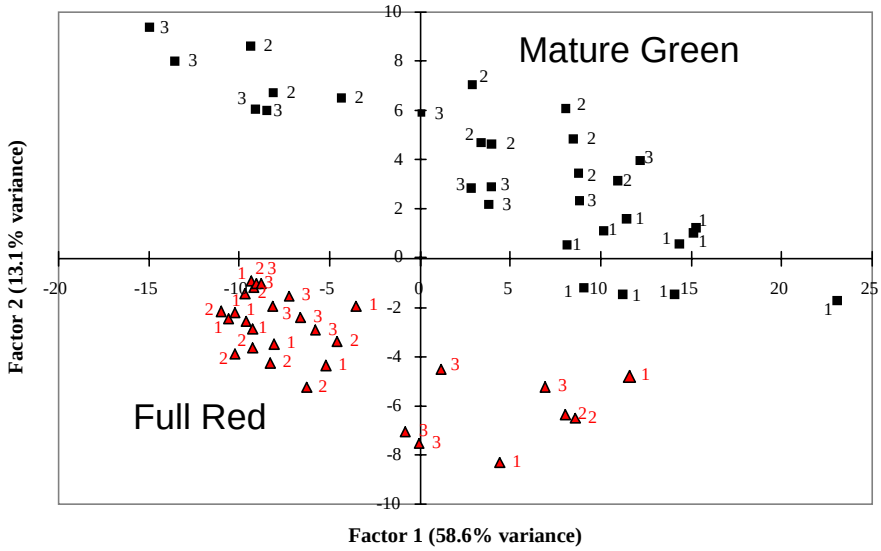


**Fig. 1**. PCA of PyMS data showing the clustering of mature green fruit (squares) and full red fruit (triangles). Three fruit were analysed for each ripeness stage (labelled as 1–3 for each group) and 10 replicate samples of each fruit were analysed. The separation between the two stages of ripeness is clear, however the variation within samples can be seen.

clear discrimination between the treatments indicating significant differences in fruit biochemistry depending on the method of ripening employed. The use of this approach may prove invaluable in addressing such problems as fruit quality and post-harvest stability.

It is well documented that growing conditions profoundly affect not only fruit yield but also fruit quality. Hence the approach of metabolic fingerprinting was adopted to study the effect of salt treatment on the biochemical profiles of fruits grown under control and 0.4% NaCl treatments. Prior to these analyses, the tomato variety

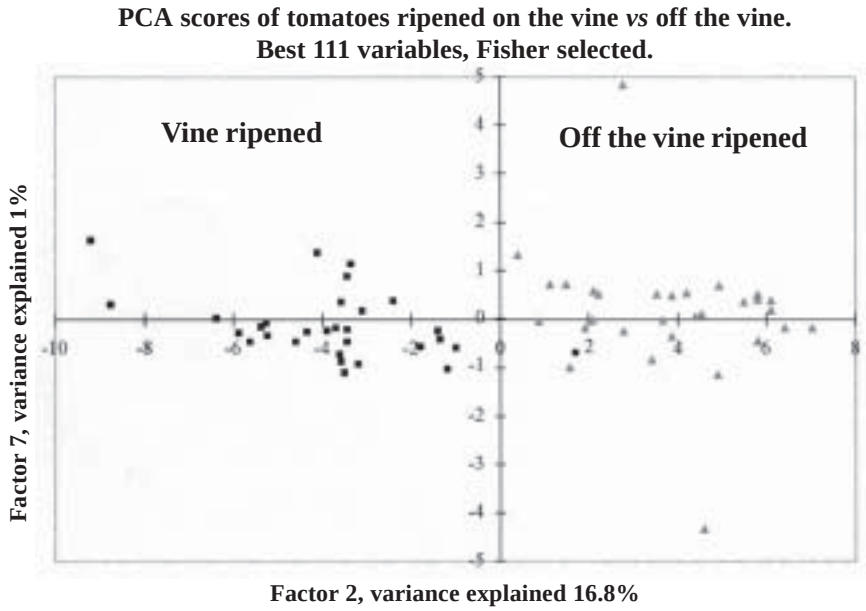


**Fig. 2**. PCA plot derived from PyMS data showing the separation of full red fruit ripened on and off-the-vine.

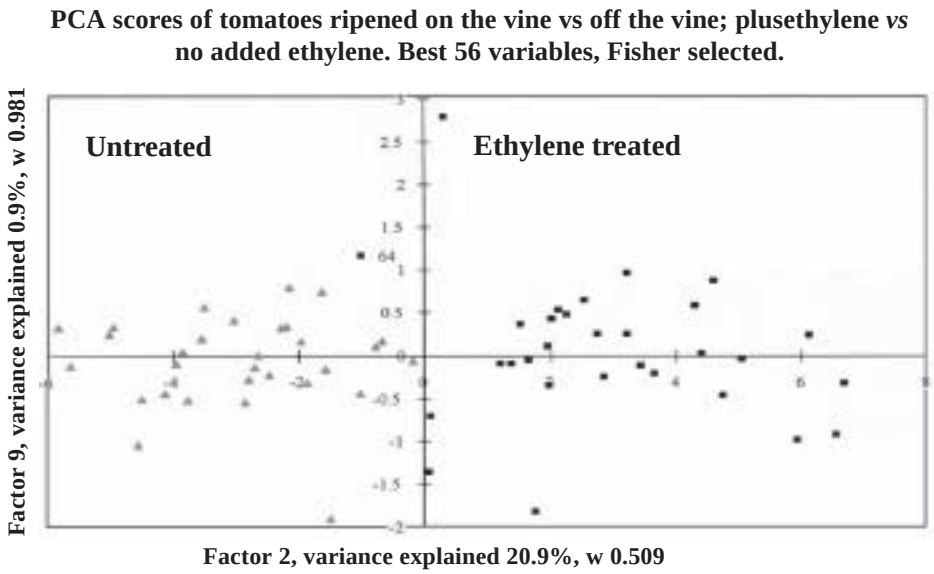


**Fig. 3**. PCA plot derived from PyMS data showing the separation of two classes of fruit both ripened off-the-vine, one set ripened with the addition of 200 $\mu l.l^{-1}$ ethylene (triangles) and the second ripened minus exogenous ethylene (squares).

Edkawy was screened for salt tolerance using classical growth analysis. Although the potential of Edkawy as a salt tolerant variety had previously been outlined (Mahmoud et al., 1986a and b), the responses to salt stress are not homogeneous and irrigation system, nutrient solution composition and other external factors all contribute to the ultimate response of plants to salt treatment. In terms of Relative Growth Rate (RGR) and Net Assimilation Rate (NAR), there was no significant difference between control and salt-treated plants (Table 1). The screening results supported the previous work that Edkawy displays salt-tolerant attributes.

Table 1. The effect of 0.4% NaCl w/v on the relative growth rate (RGR), net assimilation rate (NAR) and fruit yield of Edakwy when grown in a hydroponic drip irrigation system. The growth analysis data were collected over a two-week period, 7 days after the initial salt application. Mean of five replicate plants per treatment. ns = non significant. Fruit were harvested when fully ripe from the 1st and 2nd trusses over 26 days. BER = blossom end rot.

| | Control | Saline |
|---|---|---|
| RGR, $d^{-1}$ | 0.1381 | 0.1139 (ns) |
| NAR, $gd^{-1}$ | $6.1x10^{-4}$ | $5x10^{-4}$ (ns) |
| % Total yield | 57 | 43 |
| Mean fresh weight, g | 151.4 | 109.7 |
| Mean size class | 4.4 | 5.1 |
| % BER | <10 | 50 |

Defining susceptibility and tolerance to salinity is difficult as there is no clear cut-off point between the two attributes. The response of a plant to salinity is affected by many different factors including growth stage, magnitude and duration of the stress, and climatic conditions. Although it is important to assess the effect of salinity on vegetative growth, ultimately it is the effect of increased salinity on fruit yield and quality that is of economic importance.

Table 1 shows the fruit yield and blossom end rot (BER) occurrence in Edkawy grown under control and saline conditions. All fruit were harvested from the 1st and 2nd trusses over a 26 d period. Salinity had no significant effect on the number of fruit harvested with 43% of the total fruit yield harvested from salt-treated plants compared with 57% in the controls. However, marked differences were recorded between the two treatments when comparing the average fruit weight and size class. Salinity resulted in a decrease in fresh weight and size class (Table 1). However, the magnitude of the decrease was not as great as that reported for other tomato varieties, confirming the salt tolerant attributes of this variety.

The tissue extracts of fully ripe fruit samples from control and salt-treated plants were analysed using FT-IR. FT-IR provides rich and discriminating spectral information but also allows easier sampling loading and faster analysis time than PyMS and enables high-throughput screening of crude extracts. The FT-IR spectra all show

broad and complex contours and it is difficult to identify key discriminatory features by eye (Fig. 4). Such spectra readily illustrate the need to employ chemometric techniques for their analysis. Genetic Programming (GP) produces readily interpretable mathematical rules that enable identification of wavenumbers selected to perform classification. An analysis of which spectral variables, in terms of absorbances at particular wavenumbers, were selected showed that there were few regions of the spectra that were consistently being used to form the different GP models (Fig. 5). In particular, the spectral region covering 2270 to 1960 $cm^{-1}$ was used by most of the rules, and all the best performing models were based on a few small but distinct features within this critical region. The absolute differences between saline and non-saline grown samples in this region are very small and so would not have been selected by other types of analysis such as producing a difference spectrum. Screening of spectral libraries (HyperChem 5.1, IR Mentor Pro2 Bio-Rad) indicated that the only biochemically reasonable functional groups that absorb strongly in this critical part of the IR spectrum are acetylenes (R–C≡C–R′) and cyanides or nitriles (R–C≡N). Acetylenes have a second characteristic vibration at approximately 3300 wavenumbers, a region which was not used by any of the GP derived rules. Therefore the most likely candidate chemical moiety being identified by the predictive models as characteristic for tomatoes grown under saline conditions is a cyanide or nitrile group.

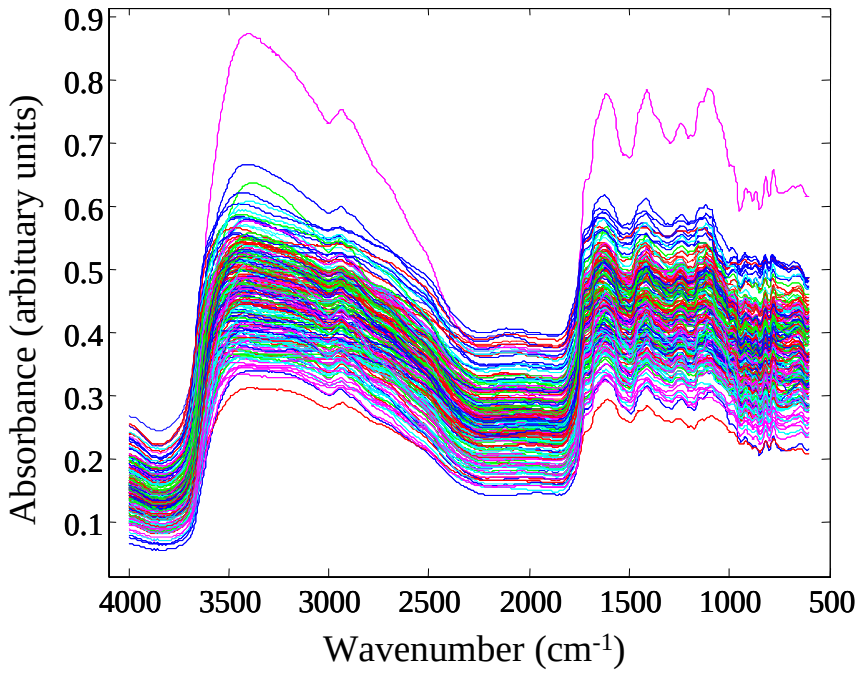


**Fig. 4**. FTIR spectra of Edkawy fruit samples.

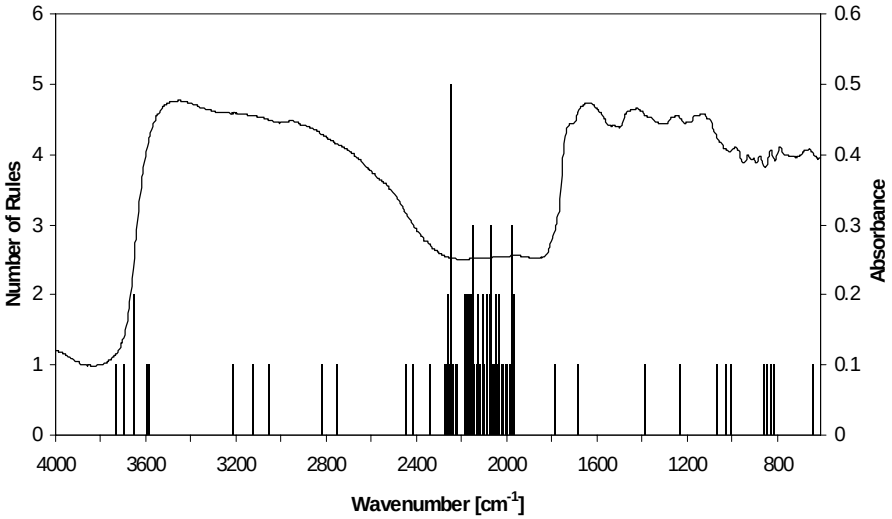


**Fig. 5**. The frequency of variable selection by 30 GP rules (histogram) with reference to a representative FT-IR fruit tissue spectrum. The region from 2270 to 1960 $cm^{-1}$ can be seen to be important for producing good predictive GP rules for the discrimination between control and salt-treated tomato fruit.

Nitrile and cyanide groups occur in many compounds found in plants. Cyanide groups often result from the detoxification of hydrogen cyanide (HCN) which is toxic to biological systems if allowed to accumulate. HCN is a by-product of ethylene synthesis (Fig. 6), the biosynthesis of which is known to increase in plants in response to stress and during the ripening of climacteric fruit such a tomato (Yang and Hoffman, 1984). It has previously been reported that tomato plants grown under saline show enhanced ethylene production (Mizrahi, 1982) with a consequent increase in HCN production. It can be hypothesised that the GP selected variables correspond to small spectral differences due to changes in the concentration of cyanide-containing compounds. Using the GP models in conjunction with knowledge of a biological system, a preliminary identification of important biochemical differences between samples can be made

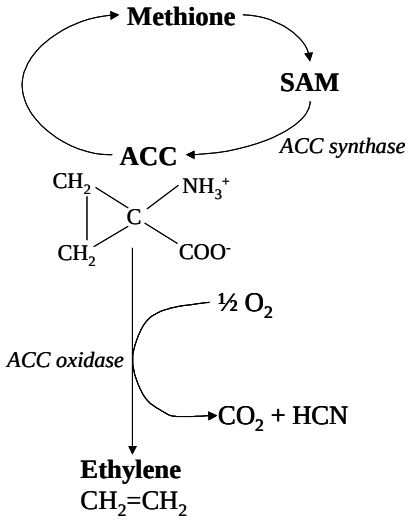


**Fig. 6**. A summary of the ethylene biosynthetic pathway showing the formation of hydrogen cyanide (HCN) during the conversion of ACC to ethylene. ACC = 1-aminocyclopropane-1-carboxylic acid. Adapted from Yang and Hoffman (1984).

providing direction for future biochemical investigations such as metabolite target analysis and metabolite profiling.

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