ACCELERATED COMMUNICATION

Rapid Screening Assay of Cyanide Content of Cassava

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A rapid, inexpensive screening assay was developed in order to measure the cyanide content of cassava (Manihot esculenta Crantz) tubers. A small disc of parenchyma tissue cut with a cork borer or alternatively grated tissue was placed in a stoppered glass tube and the hydrogen cyanide liberated produced a blue colour on a filter paper previously spotted with a drop of tetra-base [4,4’-methylenebis-(N,N-dimethylaniline)] and cupric acetate. The intensity of the blue colour which developed within one hour was rated visually on a graded scale from 0 to 5. The correlation coefficient between samples accurately analysed for total cyanide and also tested using the rapid assay was 0.77.

Keywords: Cyanide content; cassava; Manihot esculenta; linamarin; cyanogenic glycosides.

INTRODUCTION

Cassava (Manihot esculenta Crantz) is the third most important food source in the tropical world after rice and maize and is the most important food in the humid and semi-humid tropics of Africa. Cassava tubers and leaves contain cyanogenic glucosides, mainly linamarin and small amount of lotaustralin (Bradbury and Holloway, 1988). The cyanogenic glucosides and linamarase are physically separated from one another in the plant and disruption of the tissue allows the enzyme linamarase to come into contact with the former and catalyse the hydrolysis. Thus linamarin is hydrolysed to acetone cyanohydrin which further breaks down to acetone and hydrogen cyanide.

Hydrogen cyanide is a lethal poison at a level of 0.5–3.5 mg HCN/kg body weight (Montgomery, 1980; Solomonson, 1981). The parenchyma of cassava tubers contains cyanogenic glucosides equivalent to 0.2–100 mg HCN/100 g fresh weight (Bradbury and Holloway, 1988; Bradbury et al., 1991), hence high-cyanide varieties of cassava present a considerable hazard to human health, even though post-harvest treatments including cooking reduce the cyanide content. As well as the possibility of acute intoxication and death, regular exposure to sublethal amounts of cyanide may exacerbate goitre and cretinism (Delange et al., 1983) and cause tropical neuropathy (Osuntokun, 1981) and epidemic spastic paraparesis (Rosling, 1987), which is also called konzo (Howlett et al., 1990).

A simple rapid screening method for cyanide in cassava is important for cassava breeding programmes. One method used at the International Institute for Tropical Agriculture (IITA), utilizes sodium picrate paper that is suspended above a leaf disc in a sealed glass tube (Guignard, 1906; Sadik et al., 1974; Cooke et al., 1978). The HCN liberated causes a colour change from yellow to red-brown.

One problem with this method is the poor correlation between the cyanide content of leaf and tuber. Cooke et al. (1978) found a significant correlation (P<0.05) between picrate leaf score and root cyanide content (based on fresh weight of peeled root) for 108 clones (correlation coefficient r = 0.22). The correlation was improved when the root cyanide content was calculated on a dry weight basis (P about 0.01, r = 0.36). A similar correlation coefficient was obtained by Dr. M. Bokanga by calculation of the data of de Bruijn (1971). On the other hand, Mkpong et al. (1990) found that the average linamarin content of leaves from low and high cyanide varieties of cassava was essentially the same. A second problem is that the picrate method is unreliable as a quantitative method for cyanide (Williams and Edwards, 1980; Fukuba and Mendoza, 1981; Izomkun-Etiobhio and Ugochukwu, 1984).

Another method (Feigl, 1954; Esquivil and Maravalhas, 1973) uses benzidine and copper acetate, but this was abandoned because of the carcinogenic character of benzidine (Feigl and Anger, 1966). The latter authors replaced the benzidine spot test by tetra-base [4,4’-methylenebis-(N,N-dimethylaniline)] and copper ethylacetoacetate in chloroform and this method was used by Tantiswe et al. (1969) on plant material. We have further developed this method using tetra-base in acetone and cupric acetate in acetic acid: water as a rapid screening assay for cyanide in tubers and leaves.

EXPERIMENTAL

Materials. Tetra-base was obtained from Kodak (Eastman Kodak, Rochester, NY) and recrystallized from ethanol. Cupric acetate (laboratory grade reagent) was obtained from May and Baker, Dagenham, UK. Cupric ethylacetocetate

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was prepared from acetooacetic acid ethyl ester (Sigma Chemical Co., St. Louis, USA) and cupric acetate (Vogel, 1989).

**Methods.** Fresh cassava tubers and leaves were obtained from plants grown in a glasshouse in Canberra. Tubers were washed and air-dried. Because of the relatively small longitudinal gradient of linamarin concentration from the proximal to the distal end of the tuber, one circular section of thickness 2–5 mm cut across the peeled midsection of the tuber was used (Cooke, 1978; Bradbury et al., 1991). Care was taken to remove the cortex layer. Since there is a much steeper gradient of concentration of linamarin from the periphery (high) to the centre (low) of the tuber (Cooke, 1978; Kojima et al., 1983) two different methods of sampling the circular sections were used as follows:

1. For small tubers of diameter up to about 40 mm a reasonable sample was obtained by cutting a circular disc of tissue out of the section using a cork borer of diameter 21 mm placed at the edge of the section.

2. To obtain accurate sampling of the section cut from the tuber, either the whole section, or a suitably sized segment cut from it, was grated and the grating material thoroughly mixed.

Either the 21 mm diameter disc (Method 1) or a spoonful of the grated material (Method 2) was placed in a glass tube of internal diameter about 23 mm and length 50 mm sealed with a plastic cap. A rectangular strip of filter paper (about 10 mm x 45 mm) was held between the cap and the glass tube with about 35 mm hanging inside the tube. The filter paper had previously been spotted near the end with one drop of the indicator solution (see below) and the solvent allowed to evaporate completely before insertion into the tube. This required not less than 30 min at 20°C; use of damp strips of paper gave slower colour development. The indicator solution (prepared freshly each day) was obtained by mixing equal volumes of 0.3% (w/v) tetra-base in acetone and 0.3% (w/v) cupric acetate in 15% acetic acid in water. Each of these 0.3% solutions were stable for months when kept separately. Use of more dilute solutions (0.15%) caused slower colour development.

A blue colour developed and intensified over a period of 5–60 min at room temperature, depending on the amount of HCN liberated from the cassava disc. Six levels were distinguished as follows—0: no colour (obtained with a blank test containing no cassava disc), 1: very low intensity (very pale blue), 2: low (pale blue), 3: medium blue, 4: moderately high, 5: high (blue-purple). Using sampling Method 1, in order to distinguish between samples in the high range (10–100 mg HCN/100g fresh weight), the colour intensities were compared after about 15 min at 20°C, whilst for the low range (<10 mg HCN/100g fresh weight), a time of 60 min at about 20°C, or 15–30 min at 27°C, was required. In all cases the colour intensified with time and after 16 h differences between different samples were largely eliminated. Early tests were made with cupric ethylacetoacetate (Feigl and Anger, 1966), but cupric acetate was found to be equally effective and was used in all subsequent work, because of its cheapness and ready availability. However, with cupric acetate, but not with cupric ethylacetoacetate, there is slow formation of a blue colour over about 16 h at 20°C, even in the absence of HCN. When grated samples (Method 2) were compared with discs (Method 1) using the same sample of cassava, it was found that the latter were normally 1–2 levels lower in colour than the former. Thus it was important not to mix up the two methods in the one series of experiments.

The total cyanide content of cassava samples was also obtained using the recently developed acid hydrolysis method of Bradbury et al. (1991).

**RESULTS AND DISCUSSION**

According to Feigl and Anger (1966) the tetra-base is oxidized to a blue conjugated product by the copper (ii), which is reduced to copper (i), in a reaction that involves cyanide. The original technique used cupric ethylacetoacetate, which has been replaced in the present method by the more readily available and cheaper cupric acetate. Also chloroform has been replaced as a solvent by less toxic and cheaper acetone: acetic acid: water.

The blue colour intensified with time, due to the continuing liberation of HCN by the hydrolysis of linamarin (catalysed by linamarase) and the breakdown of unstable acetone cyanohydrin to HCN and acetone. Thus, at any given time after starting the experiment, the intensity of colour depended on the rate of liberation of HCN, which in turn depended on (1) the area of cut surface, (2) the temperature, (3) the amount of linamarase and possibly hydroxynitrile lyase present and (4) the amount of linamarin in the sample. As already mentioned, increasing the area of the cut surface by grating (Method 2) increased the colour intensity. A series of parenchyma samples made with disc thicknesses of 1–8 mm showed that there was no appreciable difference in colour intensity for thicknesses between 2 and 5 mm. Thus, parenchyma discs of thickness about 3 mm were used.

We have recently shown that the rate of autolysis of linamarin in cassava parenchyma tissue increased with increase in linamarase content (Bradbury and Egan, 1991), hence variation in the linamarase content (and possibly also the amount of hydroxynitrile lyase) from sample to sample must affect adversely the accuracy of the test method. The extent of this effect could perhaps be ascertained by accurate measurement of the linamarin and linamarase contents of tissue which was also subjected to the rapid assay. As shown in Table 1 we have compared the linamarin content by the acid hydrolysis and rapid assay methods, but have not measured the linamarase content. There is a reasonably good correlation between the results of the rapid assay and the total cyanide content determined by acid hydrolysis ($r = 0.77$ for 16 samples containing from 0.8 to 11 mg HCN/100g fresh weight).

**Sampling of cassava plants for cyanide**

The presently used picrate leaf test for cyanide (Sadik et al., 1974; Cooke et al., 1978) is based on a correlation between leaf cyanide and tuber cyanide. Although a significant degree of correlation was claimed by Cooke et al. (1978) there is increasing evidence that the correlation is not good enough for the leaf method to give a reliable indication of tuber cyanide content. Since plants cannot be sampled until they are mature enough to produce tubers, it is necessary to sample tuber parenchyma directly. The two methods of sampling the tubers proposed here should not be used indiscrimina-
Comparison of the tetra-base method with the picrate method

The picrate method is less sensitive than the pyridine–pyrazolone (Ikediobi et al., 1980) or the pyridine–barbituric acid methods. We have tested both methods on leaf discs (moistened with a drop of toluene; Sadik et al., 1974), cortex and parenchyma tissue discs. With low cyanide parenchyma discs the picrate method gave only a very slight colour change, whereas tetra-base gave good results, hence the latter is more sensitive, in agreement with Brinker and Seigler (1989). The tetra-base method gives more rapid colour development (1 h) than the picrate method (4 h) and it is easier to distinguish the levels of colour from white to blue than from yellow to red-brown. The tetra-base method gives much better correlation with a quantitative method ($r = 0.77$) than does the picrate leaf test ($r = 0.34$), although the low value of the latter is partly due to poor correlation between leaf cyanide and tuber cyanide in the same plant. The tetra-base method uses only small amounts of tetra-base which is not expensive (US$40 per 100 g) and inexpensive cupric acetate. At least 100 tests can be made for one cent.

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