THE CHLOROFORM TEST—A STUDY OF ITS SUITABILITY AS A MEANS OF RAPIDLY EVALUATING FERMENTING PROPERTIES OF CLONES

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The Chloroform Test was described to the writer by Dr. E. M. Chenery, Director of the East African Tea Research Institute, during his visit to Ceylon in October, 1963. The test is used in East Africa as a rapid means of detecting bushes with poor fermenting properties. In East Africa about 20% of the bushes in an average field have been found to be poor fermenters by this test (Dr Chenery, personal communication).

The Chloroform Test appears to be an old one, but interest in it has been revived recently by Bendall (1959). In theory the Chloroform Test works as follows: The leaf to be tested for ability to ferment is placed in an atmosphere of chloroform vapour. The chloroform vapour kills the tissue and causes the cellular membranes to become permeable. When this happens the polyphenols, or flavanols, present in the vacuole of the cell move by diffusion into the cytoplasm of the cell where they come in contact with the enzyme polyphenol oxidase. On contact the enzyme oxidizes the polyphenols changing them into brown coloured substances. This process is equivalent to tea fermentation and the extent of the reaction can be judged visually as the amount of brown colour developed in a specified unit of time.

An investigation into the reliability of the Chloroform Test was undertaken in order to assess its usefulness in rapidly evaluating the fermenting properties of tea bushes.

Methods

The Chloroform Test:—The tests were carried out in glass stoppered test tubes, 20 × 130 mm, but any other glass container which can be closed may be used. The tubes are prepared by placing a small wad of cotton wool in the bottom and adding about 10 drops, about 0.4 ml., of chloroform. The tubes are closed after adding the chloroform and allowed to stand for five minutes to allow the atmosphere within the tubes to become saturated with chloroform vapour.

It is important that the level of liquid chloroform does not come above the cotton wool. If the leaf comes in contact with liquid chloroform anomalous results will be obtained and the test will be invalidated.

The flush, *i.e.* the apical bud and two leaves of all the new shoots on the bush, is collected from the bush to be tested. From this material two or three of the first leaves are selected for the test. Uniform leaves which are representative of all the first leaves should be selected. These leaves are pinched off the shoots and then they are placed in individual tubes apex down. The test tubes are again stoppered and the time recorded. The closed test tubes are then allowed to stand at room temperature for ninety minutes. It may be advisable to vary the time from location

to location depending on the prevailing temperatures. In warmer climates shorter test periods will be required whereas in cooler climates longer periods may be necessary. The set-up used for this test is shown in Figure 1.

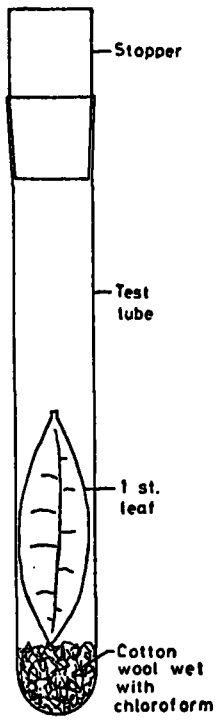

Figure 1. A Typical Set-up for the Chloroform Test. Other similar containers may be used.

At the end of ninety minutes the tubes are examined and the colour of the leaves is noted. The following colours, in order of their appearance, should be differentiable:

(1) Green (G)
(2) Green but veins golden (YV)
(3) Greenish golden (GY)
(4) Golden (Y)
(5) Golden-brown (YB)
(6) Brown (B)

The closer the colour of the leaf to brown, following the above order, after ninety minutes, the better the fermenting properties of the bush tested.

The Mincing Test:—Flush from the test bush is minced in a mincing machine. The minced leaf is placed in a dish and spread out about one-fourth inch deep. After one hour the colour of the minced leaf is noted using the same colour scale as used in the Chloroform Test. This test approximates very closely the results obtainable in commercial practice as regards fermentation rates.

Polyphenol Oxidase Assay:—This assay was carried out essentially as described by Sreerangachar (1943). In brief, an acetone powder is prepared from the fresh material to be tested. The acetone powder is then incubated in a catechol-buffer solution for 15 minutes. The amount of catechol oxidized is determined by titration; this being a measure of the enzyme activity.

Total Flavanols:—The vanillin test described by Swain and Hillis (1959) was used to estimate total flavanols. These are the compounds within the tea leaf which are acted on by the enzyme polyphenol oxidase during fermentation.

Results

Collection of flush from different clones was made on several occasions and the Chloroform Test was carried out on the material collected. For comparison, each sample was also tested by the Mincing Test and its polyphenol oxidase activity and total flavanol content were measured. Some of the results obtained are shown in Table 1. These are typical of all the results obtained in this investigation and they serve to illustrate the findings made very well.

The first and most important finding is that the Chloroform Test and the Mincing Test gave identical results. This establishes the validity of the Chloroform Test as a rapid means of evaluating the fermenting properties of a bush. However, comparison of the results obtained on two different dates shows that the fermenting properties of a bush are not constant with time. The rate of fermentation in all clones was markedly slower on 10th December than on 17th December as shown by the Chloroform Test. This is undoubtedly caused by the lower polyphenol oxidase activity on the former date which can also be seen in Table 1.

TABLE 1.—*Comparison of twelve clones on two dates for fermenting properties by the chloroform test and other ancillary biochemical quantities.*

Clone	10th December 1963					17th December 1963				
	Chloroform Test & Mincing Test		*Polyphenol Oxidase Activity*		*Total Flava-nols*	*Chloroform Test & Mincing Test*		*Polyphenol Oxidase Activity*		*Total Flava-nols*
	Colour	*Rank*	*Activity Units*	*Rank*	*Units*	*Colour*	*Rank*	*Activity Units*	*Rank*	*Units*
TRI. 777	B	1	12.1	1	24.5	B	1	19.4	1	23.5
CY.9	YB	2	8.5	5	24.0	GB	2	10.7	8	22.0
TRI. 2024	YB	3	11.4	2	27.0	Y	5	17.9	2	29.0
KEN. 16/3	YB	4	6.1	11	24.0	Y	4	12.2	6	22.5
TRI. 2026	Y	5	7.4	9	24.0	Y	6	10.9	7	24.0
DT. 1	Y	6	7.4	8	25.5	Y	3	13.2	3	27.0
TRI. 2025	GY	7	10.5	3	27.0	GY	10	12.5	5	29.5
TRI. 2023	YV	8	8.3	6	23.0	GY	8	9.5	10	27.0
PA. 22	YV	9	7.7	7	25.0	Y	7	9.5	9	26.5
TK. 48	YV	10	6.2	10	22.0	YV+	11	9.0	11	25.5
UH. 9/3	YV-	11	9.5	4	30.0	GY	9	13.0	4	30.5
CV. 5.B. 1	YV-	12	2.8	12	25.5	YV	12	6.8	12	26.5

It is noteworthy that the relative rank of the clones remains approximately the same from date to date in spite of wide variations in the rates of fermentation found on different dates. The agreement is not perfect but it is close enough to be a useful characteristic.

Examination of the results also shows that fermentation rate is not entirely dependent on the level of polyphenol oxidase activity. The clones TRI.2025, UH.9/3, CY.9 and KEN.16/3 serve to illustrate this. That is, clones TRI.2025 and UH.9/3 are rather poor fermenters in spite of the fact that they possess high levels of polyphenol oxidase. On the other hand, clones CY.9 and KEN. 16/3 are

good fermenters even though they possess rather low levels of the enzyme. This is rather surprising in view of the fact that the most obvious changes occuring during fermentation, *i.e.* the oxidation of the tea flavanols, are brought about by this enzyme. It may be mentioned here that the results obtained with the Chloroform Test have also been confirmed by tasters tasting experimental teas made by the Technology Division from these same samples.

Further examination of Table 1 reveals that the level of flavanols themselves is also not the deciding factor in determining the fermentation rate. Notice especially clone UH.9/3 which has high polyphenol oxidase activity and a high flavanol content but is still a poor fermenter. The answers to these problems are not known at present but that does not affect the usefulness of the Chloroform Test directly.

Discussion and Conclusions

The results show clearly that the Chloroform Test is a useful means of rapidly evaluating the fermenting properties of tea bushes. Furthermore, the test has been found to be very simple and easy to use adding to its value.

However, certain limitations are also apparant from the results of this investigation and therefore certain precautions should be taken when using this test in order to be able to arrive at accurate conclusions. These may be enumerated as follows:

(1) Test the same bush on several days over a period of several weeks before coming to a final decision.

(2) In every test include one or more standard clones of known fermenting properties for comparison.

The test is very easy to carry out and it requires only a minimum of equipment. Reliable results should be obtainable with only a limited amount of practice provided one is careful to follow the procedure given and to observe the precautions listed above. The test should be useful to interested planters, particularly in clonal selection work.

Acknowledgments

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