BIOLOGIA PLANTARUM (PRAHA)

5 (2) : 310—317, 1963

Quantitative Determination of Cyanogenesis in Plants

VÁCLAV POKORNÝ

Institute of Genetics, Faculty of Natural Sciences, Charles University, Institute of Experimental Botany, Czechoslovak Academy of Sciences, Praha*

Received October 5, 1962

Kvantitativní stanovení kyanogenních vlastností rostlin

V práci je popsána metoda vhodná k sériovým stanovením pro genetické pokusy. Kolorimetrické stanovení HCN (1fenyl-3methyl-5pyrazolonem a pyridinem, fenolftalinem nebo Na-pikrátem) dovoluje zpracovat vzorky uvolňující 0,2μg až 20 μg HCN. Před štěpením glykosidu možno provést stanovení obsahu oleje v semenech. Analysa variance kyanogenních vlastností jednotlivých semen dvou odrůd lnu s průměrným obsahem 0,4 a 0,9 μg HCN ukazuje, že je možno zachytit rozdíly mezi rostlinami i rozdíly mezi tobolkami téže rostliny.

Summary

A method suitable for serial determinations in genetic experiments is described. Colorimetric determination of HCN (by 1-phenyl-3-methyl-5-pyrazolone and pyridine, phenolphthalin or sodium picrate) facilitates processing of samples releasing 0.2 μg to 20 μg of HCN. Before hydrolysis of the glycoside the oil content in the seeds can be determined. Variance analysis of the cyanogenic properties of the particular seeds of two sorts of flax with an average HCN content of 0.4 and 0·9 μg shows that the differences between plants and between capsulae of the same plant can be determined.

Introduction

In the species of plants forming cyanogenetic glycosides four types are encountered

1. The plant contains the glycoside and simultaneously also the corresponding hydrolysing enzyme.

Address: Na cvičišti 2, Praha-Dejvice

2. The plant contains the glycoside only.
3. The plant contains the hydrolysing enzyme only.
4. The plant contains neither glycoside nor enzyme.

The typical cyanophoric properties described by MIRANDE (1909) are shown by the plants only in the first case. Through the influence of the vapours of certain substances (chloroform, ether, toluene etc.) or on injury and wilting, hydrolysis of the glycoside occurs and the plant releases HCN.

Cleavage of the glycoside with the aid of a foreign enzyme from another plant can also be performed with the second group of plants.

With the third type of plants obviously only the enzyme can be evaluated.

All four phenotypes mentioned can be found in the population of the same type or sort of culture plants, as was observed by CORKILL (1940) with *Trifolium repens* L.

In other types, the glycoside is found predominantly only in certain organs, for example in the leaves of *Sambucus nigra* L. (BOURQUELOT, DANJOU, 1905). A characteristic and essential part of the quantitative evaluation of the cyanophoric properties of plants, therefore, is represented by the problem of the release of HCN. Absorption and determination of HCN is predominantly an analytical problem.

Method

The methods most widely used for the determination of cyanogenetic glycosides in plant material were reviewed by ROSENTHALER (1932) and SEIFERT (1955). Most authors recommend the release of HCN with the aid of the enzymes contained in the sample. HCN is transferred into the absorption solution with the aid of a stream of air or nitrogen in contrast to the older methods employing steam. Such a method adapted in detail to the material was described by LÜEDTKE (1952, 1953) for flax seeds or seedlings. Samples of several grams are usually employed, amounts lower than 1 g are only used exceptionally. For serial analysis of particular seeds, leaves etc. another method must be chosen.

As will be mentioned later, release of HCN first proceeds rapidly. For example more than 50% of HCN is released within 1 hr. from the cotyledons of flax. Standing for several hours before removal of HCN therefore cannot be recommended since an equilibrium is established which inhibits further cleavage of the glycoside. A comparatively large volume of the reaction mixture also is unfavourable since the HCN concentration in the solution is higher than in the air above the solution. Detailed data were given by ROBBIE (1946). It therefore will be better to preserve a small volume and to remove the HCN released right from the start. For the transfer of HCN released into the absorption solution first vessels were employed which are based on the principle suggested by KRÜGER (1935). Forced air circulation in the closed space in these vessels replaced expulsion of HCN by aeration. The vessels are shown in Fig. 1.

In Fig. 2 the amount of HCN absorbed in dependence on time is compared on forced circulation (1) and with free diffusion (2) in a stationary vessel. The amount of HCN absorbed in 12 hr. in both cases was taken as 100%. For evaluation of the total amount of HCN, after an adequate reaction time, free diffusion of HCN into the absorption solution is usually sufficient. The absorption conditions can be improved by a relative increase in the level of the absorption solution and by bringing the sample closer.

The following circumstances must be taken into consideration for preparing the sample and hydrolysis of the glycoside: Fresh tissue, e.g. leaves release HCN on injury or wilting. The glycoside content of flax seedlings was lowered by about 30% by air drying. It was found on the other hand that torn off leaves (*Linum usitatissimum* L., *Trifolium repens* L.) do not release even traces of HCN within 24 hours if stored in a humid atmosphere without wilting (in a closed reaction vessel with absorption liquid). The glycoside is easily hydrolysed, for example, by toluene vapours or by immersion of the sample (leaf) into toluene for about 2 minutes. Shortening of the time to 1 minute lowered the results with flax cotyledons by about 4% as compared to 2 min. immersion into toluene, if cleavage was carried out for 4 hr. only. The differences were equalized after 12 hr. cleavage. Completeness of hydrolysis is doubtful when whole organs (leaves, seeds)

are analyzed without homogenisation. Homogenisation of fresh or dried tissues should be performed after inactivating the enzymes. This, however, would complicate the method.
The following tests were therefore undertaken:

1. 20 flax cotyledons (the normal sample was 1 cotyledon) were dried after 12 hr. of HCN release by cleavage of the glycoside with its own enzyme, ground and placed in a new absorption vessel and the sample wetted with citrate buffer (pH 6). No HCN was found in the absorption liquid after another 12 hr.
2. 20 flax cotyledons were inactivated by boiling for 10 min. After drying and grinding the leaves gave a negative reaction for HCN.
3. The sample from the second test gave a strongly positive reaction after adding the sample from the first test.

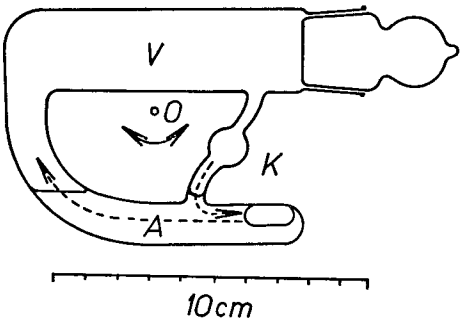

Fig. 1.

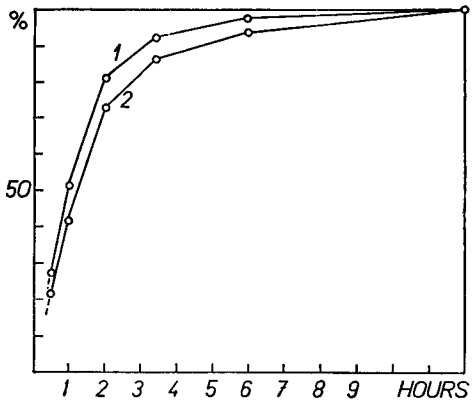

Fig. 2.

Fig. 1. Schematic design of the absorption vessel. By oscillation around axis 0 the absorption fluid A is set in motion which in one limiting position sucks up gas from the reaction space V through tube K. At the reverse movement the capillary forces holding a short column of absorption liquid in tube K propel the bubble through the total volume of the absorption fluid before returning to the reaction space V.

A sample after normal analysis therefore did not release any HCN but it contained an active enzyme. Analogous results were obtained with flax seeds which had been previously pressed and deprived of oil by extraction. Decomposition of the glycoside and release of HCN apparently occurs to a sufficient extent even without homogenisation.

The amount of HCN released by the enzymes from the sample therefore can be considered a suitable measure of the cyanophoric properties of the plant and in most cases also of the content of the cyanogenetic glycoside.

With material containing the glycoside only (*Sambucus nigra* L., certain plants of *Trifolium repens* L.) the sample can be dried and ground or homogenized. The determination is carried out after addition of the sap or extract of material from which HCN was removed or of plants containing only the enzyme. In any case the procedure is more complicated and less suitable for serial determination, especially with small samples.

For qualitative estimation of the cyanophoric properties of plants it must be pointed out that long reaction times (24 hr. or more) can lead to errors with plants containing the glycoside only. This time is sufficient for multiplication of contaminating microorganisms which can cause release of HCN. This was found with *Sambucus nigra* L. leaves.

Temperature, of course, influences the course of the reaction. At sufficiently long reaction times (12 h), however, it is not necessary to maintain higher temperatures (40° C). Normal room temperature is satisfactory.

The suitable pH lies between 5 and 6 (emulsin, linamarase). The activity of the enzyme in some cases (linamarase with *Trifolium repens* L.) decreases at pH higher than 6 (Coop 1940).

Determination of Hydrogen Cyanide

Analytical determination of HCN is generally carried out after absorption in 0·1 N NaOH. The selection of a suitable method is mainly determined by the sensitivity required. The amount of HCN released, for example, from a single flax seed is in the order of magnitude of 1 μg. From a series of colorimetrical methods for cyanide determination three methods suitable for serial analysis were chosen.

The most sensitive and specific method is that of Epstein (Epstein, 1947), somewhat less sensitive is the simple phenolpthalin method (Robbie, 1946) and the simplest but least sensitive is the picrate method.

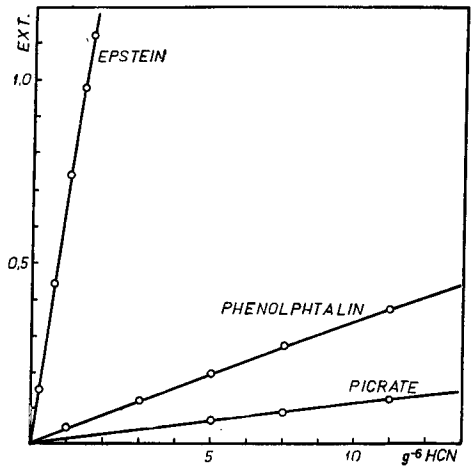

Fig. 3.

Comparison of the sensitivity of the given methods. Extinction was measured with interference filters of 620 mμ (Epstein) and 545 mμ (phenolphthalin and picrate).

The sensitivity of these three methods is compared in Fig. 3. All measurements were carried out in 5 ml test tube cuvettes on the Lange photocolorimeter UK VI. at identical volume of the reaction mixture. The filters employed are given in the legend to the diagrams.

Remarks to the particular methods.

The picrate method because of its simplicity is also employed as a qualitative test of the cyanophoric properties of the plants (Guignard, 1906).

The analytical application of the red isopurpuric acid formed with the picrate method was described in detail e.g. by Möller and Stefanson (1937).

On application of a 5 ml cuvette the procedure is: 4 ml of 1% picric acid are added to 1 ml of 0·2 N NaOH containing absorbed HCN and the mixture is heated in a boiling water bath for 10—12 min. Colorimetry can be performed immediately after cooling and adjusting the volume. Moller and Stefanson (1937) recommend measurement of absorption at 494 mμ where picrate is not yet absorbed. The amount of HCN suitable for determination in a 5 ml cuvette is 10—20 μg.

Although HCN is isolated from the sample by diffusion a nonspecific reaction also must be considered since H_2S interferes and acetone and acetaldehyde also cause colouring (Möller and Setfanson, 1937).

The phenolphthalin method was described in detail by Robbie (1946) who also cited older references. The method is based on the principle of the oxidation of colourless phenolphthalin to phenolphthalein. Quantities of HCN of 1—10 μg are suitable for the determination at a reaction volume of 5 ml and measurement in a 5 ml test tube cuvette.

Preparation of the reagent according to Robbie (1946): 50 mg of phenolphthalin are dissolved in 10 ml ethanol and after dissolving 990 ml of 0·01% aqueous $CuSO_4 . 5H_2O$ are added. The water employed for preparation of the sample or the reagent should not contain traces of copper.

At room temperature the reagent is stable for several days, in the refrigerator for several months.

Phenolphthalin can be prepared from phenolphthalein by reduction with zinc powder at boiling temperature in alkaline solution (NaOH or KOH).

The greatest disadvantage of the method is the instability of the colour formed. The transformation of phenolphthalin to phenolphthalein proceeds even after the termination of the main reaction. Conversion of unreacted phenolphthalin is greater in the presence of excess of the reagent. The reaction mechanism assumed proceeds through reduction of copper to copper/I/--cyanide. It was attempted in this work to remove copper by versene after termination of the main reaction. Only partial success was achieved. Addition of versene (EDTA-Chelaton III, Lachema) inhibits further transformation of phenolphtalin but the versene excess on the other hand causes gradual decrease of the intensity of coloration. The colour is stable for several hours only at suitable versene concentration (probably with regard to unreacted Cu··).

However, the colour remains essentially unchanged for 5—10 min. even at slight versene excess and the time of measurement need not necessarily be maintained as accurately as with the original method where measurement was required after exactly 1 min.

The results of absorption measurements of a standard containing 10 μg of HCN and different concentrations of versene and without versene are given in table I. Absorption increased by about 5% after 10 minutes without the addition of versene, on addition of 1 ml. of 0.0009 M versene the absorption changes during the same period were smaller than 0.2%. The addition of 0.001 M versene solution to samples containing up to 10 μg HCN therefore can be recommended under the reaction conditions mentioned below. The reaction is not specific (ROBBIE, 1946), but the presence of interfering substances in this sample is without effect if the isolation of HCN is carried out by diffusion in the gaseous state.

Table 1

Concentration of versene	% absorption after 1′	2′	3′	5′	10′	20′	30′
	55.8	56.2	56.5	57.1	57.4	57.8	58.3
8.10^{-4}M		56.3	56.5	56.6	56.66	56.7	56.73
9.10^{-4}M		56.0	56.07	56.07	55.8	55.3	55.1

With 5 ml of the reaction mixture the procedure is: 2 ml of 0·07 M H_3BO_3 are added to 1 m of 0·1 N NaOH containing absorbed HCN (to pH 10·5—11) and then 1 ml of the phenolphthalin reagent. After exactly 1 min 1 ml of 0.001 M versene solution (Chelaton III) is added. The absorption is measured in 5 ml test tube cuvettes using an interference filter 545 m μ.

The method of EPSTEIN (1947) is very sensitive and relatively specific. A certain disadvantage is the narrow concentration range suitable for measurement. A content of about 0.2—1.2 μg of HCN is most favourable with a 5 ml test tube cuvette. Details on the mechanism and the reaction conditions, the preparation of the reagent and the accuracy of the method are given in the paper of EPSTEIN and in Standard Methods for the Examination of Water (1955).

The method is based on the principle of the conversion of cyanide to chlorocyane by a chloramine solution. Chlorocyane further reacts with pyridine and the glutacon-aldehyde gives a blue colour with 1-phenyl-3-methyl-5-pyrazolone.

Reagents required according to the papers cited:

1) 1% aqueous chloramine T-solution, freshly prepared every day,
2) pyridine-pyrazolone reagent: 100 ml of pyridine are added to 500 ml of a filtered saturated (at 75° C) aqueous 1-phenyl-3-methyl-5-pyrazolone solution (0·1 g of bis-pyrazolone was previously dissolved in 100 ml of pyridine). The reagent is not stable and should not be older than 3 days. It is better to prepare a fresh reagent daily. A slightly pink coloured reagent does not affect the determination. The 1-phenyl-3-methyl-5-pyrazolone employed for preparing the reagent should be twice recrystallized from 95% ethanol (m.p. 127—128°). Preparation of bis-(1-phenyl-3-methyl-5-pyrazolone): 0.1 mol (17·4 g.) of recrystallized pyrazolone is dissolved in 100 ml. of 95% ethanol and 0·25 mol (25 g) of freshly distilled phenylhydrazine are added. This mixture is heated under reflux for 4 hr. The insoluble part is filtered while still hot and washed several times with 95% ethanol. The obtained bis-(1-phenyl-5-methyl-5-pyrazolone) has the m.p. > 320° C.

The method of determination can be as follows: to 1 ml of 0·1 N NaOH containing absorbed HCN, are added 1 ml of 0·2 M KH_2PO_4 and then 0·2 ml of 1% chloramine T, the vessel is rapidly closed and shaken. After 1 min 3 ml of the pyridine-pyrazolone reagent are added rapidly and

the mixture is shaken. If the sample contains CN', a pink colour appears immediately which slowly changes to clear blue. The maximum colour appears according to Epstein after 20 min at 25° C. The velocity of coloration depends on temperature and pH. The colour is stable for 30 min. after attaining the maximum. The suitable time for measurement is best determined empirically. Absorption was always measured in 5 ml. test tube cuvettes using a 620 m μ interference filter.

All three methods described are given for measurement in 5 ml test tube cuvettes without dilution, for measurement of the whole sample. The concentration ranges suitable for the particular method permit determination from about 0·2 μg to about 20 μg of HCN, provided the suitable method is selected according to the HCN content in the analyzed material.

Technical arrangement of the determination. Cleavage of the glycoside and the colour reaction was performed in 16 × 100 mm test tubes closed by a rubber stopper.

1 ml of 0·1 N NaOH (with the picrate method 0·2 N NaOH) was pipetted on to the bottom of the test tube for HCN absorption.

The sample (fresh leaf after the action of toluene, a seed deprived of fat by pressing and extraction, part of a leaf, or a weighed amount of a ground seed) was placed in a vessel made from a polyvinylchloride foil and both put into a test tube above the sodium hydroxide solution. A small amount of buffer (0·2 M Na_2HPO_4 + 0·2 M KH_2PO_4, 1 : 1) was added, if the sample was extracted seed, then the test tube was closed with the rubber stopper. Hydrolysis proceeded in a thermostat or at room temperature. It is better to leave the samples in the test tubes over night, i.e. for 16—18 hr. The samples were removed after this time and HCN absorbed in the alkaline solution was determined by one of the methods described.

The polyvinylchloride vessel employed and its arrangement in the test tube can be seen in Fig. 4.

The vessels were made from polyvinylchloride foils of 0·5 mm thickness. In the centre of a 11 × 70 mm strip a plate of 6 mm diameter and 1 mm depth was first impressed (in the cold). Both ends of the foil strip were bent in the way shown in the Figure. The sample is placed on the plate, the bent ends keep the sample in a suitable position in the test tube.

The following process proved suitable for the extraction of single small seeds: the particular seeds were first pressed between filter paper in a laboratory hydraulic press. The paper with the pressed seed for extraction was held between the threads of a spiral coiled from stainless steel wire of about 0·7 mm thickness. Thus 20 and more seeds can be extracted simultaneously. The seeds are in good condition for HCN determination. The oil content can also be determined by differential weighing before and after extraction. The seeds were extracted with petrol ether in the Soxhlet apparatus for 4 hr. immediately after pressing.

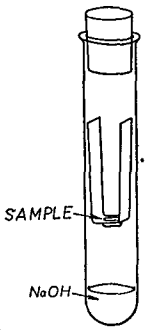

Fig. 4. Absorption vessel with sample.

Results and Discussion

The whole procedure for determination consists of these operations:

1. The fresh leaves are placed into toluene for 2 min. Seeds containing oil are pressed and extracted.

2. Cleavage of the sample is carried out in a test tube above hydroxide in a thermostat or at room temperature.

3. After removal of the sample the CN′ content is determined by one of the given methods.

Results obtained with flax are given for complete evaluation of the described determination of the cyanogenic properties of plants. 114 seeds of flax of two different varieties (Concurrent and Diadem) which were harvested in the same year, were analyzed. The seeds came from 42 capsulae from 16 plants. The results of variance analysis of the values found are given in Table III. The

amount of HCN released is expressed in μg without recalculation for the size of the seeds. HCN was determined by the method of Epstein.

Table 2
Variance analysis of the HCN content in flax seeds

Variability	$S(x - \bar{x})^2$	N	V	F
Total	13.2470	113		
between varieties	5.97561	1	5.97561	**260.21
between plants within the variety	*3.03272*	*14*	0.21662	**9.43
between all plants	0.00833	15		
between the capsulae of the same plant	*2.58527*	*26*	0.09943	**4.33
between all capsulae	11.5936	41		
Residual	1.6534	72	0.02296	

Concurrent: $\bar{x} = 0{\cdot}903\ \mu$g HCN $\quad s = 0{\cdot}1515\ \mu$g HCN
Diadem: $\bar{x} = 0{\cdot}445\ \mu$g HCN $\quad s_{\bar{x}} = 0{\cdot}02006\ \mu$g HCN
Variation coefficient $v = 22.5\%$

Variance analysis shows that not only can differences between the varieties be determined by the method described but even differencies between plants of the same variety and also differencies between the capsulae of the same plant appear highly significant. It must be emphasized that the residual variance, which was a measure for variability of the other components, does not comprize methodical errors only but also the differences between seeds from the same capsulae. This means that the methodical errors of the determination itself will be lower still.

If the cyanophoric properties of the material are charaterized by the amount of HCN released under the given conditions, then the method described is suitable for serial determinations in genetic experiments.

The decomposition of the glycoside was quantitative at sufficiently long time of hydrolysis of the material under investigation. Characterization of the individua by the amount of HCN released, however, could lead to erroneous conclusions on the amount of glycoside in this sample in the presence of any type of inhibition or deficiency of the hydrolysing enzyme.

References

American Public Health Association: Standard Methods for the Examination of Water Sewage and Industrial Wastes. — 10th Ed. 293—302, 1955 (New York).

Bourquelot, E., Danjou, E.: Sur la sambunigrine, glucoside cyanhydrique nouveau retiré des feuilles de Surreau noir. — C. R. Acad. Sci., Paris, **141** : 598—600, 1905.

Coop, J. E.: Cyanogenesis in white clover (*Trifolium repens*). III. A study of linamarase, the enzym which hydrolyses lotaustralin. — N. Z. Jour. Sci. & Tech., **22** : 71B-83B, 1940.

Corkill, L.: Cyanogenesis in white clover (*Trifolium repens*) I. Cyanogenesis in single plants. — N. Z. Jour. Sci & Tech., **22** : 65B—67B, 1940.

Epstein, J.: Estimation of microquantities of cyanide. — Anal. Chem., **19** : 277—274, 1947.

Guignard, M. L.: Le haricot à acide cyanhydrique, *Phaseolus lunatus* L. — C. R. Acad. Sci., Paris, **142** : 545—553, 1906.

KRÜGER, F.: Ein neuer Mikro-Respirationsapparat zur laufenden manometrischen Bestimmung des respiratorischen Quotienten. Z. vergl. Physiol., **21** : 249—266, 1935.
LÜEDTKE, M.: Über das Linamarin des Leinsamens und seine Bestimmung. — Biochem., Z., **322** : 310—319, 1952. Biochem. Z. Über das Linamarin des Flachsstengels. — Biochem. Z., **323** : 428/436, 1953.
LÜEDTKE, M., HAHN, H.: Über den Linamaringehalt gesunder und von Colletotrichum lini befallener junger Leinpflanzen. — Biochem. Z., **324** : 433—442, 1953.
MIRANDE, M.: Influence excercée par certaines vapeurs sur la cyanogenèse végétale. — C. R. Acad. Sci., Paris, **149** : 140—142, 1909.
ROBBIE, W. A.: The quantitative control of cyanide in manometric experimentation. — J. Cell. comp. Physiol., **27** : 181—209, 1946.
ROSENTHALER, L.: Blausäureglucoside (Oxynitrilglucoside). In KLEIN, G.: Handbuch der Pflanzenanalyse, Bd. **3** : 1036—1057, 1957 (Wien).
SEIFERT, P.: Blausäureverbindungen. In PEACH K., TRACEY M. V.: Moderne Methoden der Pflanzenanalyse, Bd. **4** : 676—688, 1955 (Berlin).

В. Покорны (Генетический институт факультета естествознания, Карлов университет, Институт экспериментальной ботаники ЧСАН, Прага)

BIOLOGIA PLANTARUM (PRAHA) 5 (4): 310—317, 1963

Количественное определение цианогенных свойств растений

В работе описан метод, подходящий для серийных определений в генетических опытах. Колориметрическое определение HCN (1-фенил-3-метил-5-пиразолоном и пиридином, фенолфталином или Na-пикратом) позволяет обработать пробы, выделяющие 0,2 μg — 20 μg HCN. До расщепления гликозида возможно установить содержание масла в семенах. Анализ варианции цианогенных свойств отдельных семян двух сортов льна с средним содержанием 0,4 и 0,9 μg HCN указывает на то, что возможно определить различия между растениями и различия между коробочками одного и того же растения.