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SCUOLA DI DOTTORATO DI RICERCA

Scienze dei Sistemi Agrari e Forestali

e delle Produzioni Alimentari

Indirizzo Produttività delle Piante Coltivate

Ciclo XXIII

METABOLITES PRODUCED DURING DIFFERENT GROWTH CONDITIONS OF

Sorghum bicolor (L.) Moench

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Alla mia famiglia

Le tre regole di lavoro:

1. Esci dalla confusione, trova semplicità.

2. Dalla discordia, trova armonia.

3. Nel pieno delle difficoltà risiede l'occasione favorevole

A. Einstein

Giovanni G. Barmina. Metabolites produces during different growth conditions of Sorghum bicolor (L.) Moench. Tesi di dottorato in Produttività delle Piante coltivate, Università degli studi di Sassari.

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Giovanni G. Barmina. Metabolites produces during different growth conditions of Sorghum bicolor (L.) Moench. Tesi di dottorato in Produttività delle Piante coltivate, Università degli studi di Sassari.

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INTRODUCTION

Secondary metabolites

The secondary metabolites play a role in the plant if not essential, however, important. In the last decade, there has been an increased awareness that many secondary metabolites play fundamental roles as defences against predators and competitors, infochemicals and allelogens. The synthesis and accumulation of secondary metabolites in plants can be induced by biotic and abiotic stress. The biotic stress is the attack from herbivores and pathogens in the plant that release chemicals that stimulate the production of secondary metabolites, while the abiotic stress is due to the surrounding environment such as the proximity of other plants, temperature, pH, evaporation, fungicides, antibiotics, heavy metals. These factors are considered elicitors, and they are stimulators for the production of secondary metabolites (Vasconsuelo and Boland 2006).

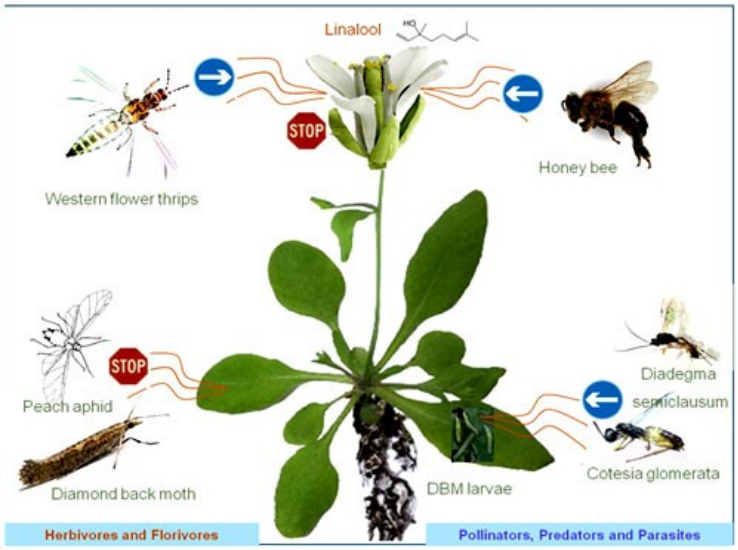


Fig. 1 Interaction plant – environment, mediated by secondary metabolites (Jongsma, 2010)

An example of defense against herbivores has been observed in sorghum plants. That plant synthesizes the cyanogenic glucoside dhurrin (p-hydroxymandelonitrile-β-D-glucoside).

This compound is present in high amounts when that plant is young, and this can be due at the fact that the plant use it as defence for protect its youngest developing tissue and the lowest part of the stem because these parts are vital for the plant's growth. These functions can be classified as mediators in the interaction

of the plant with its environment, such as plant–insect, plant–microorganism and plant–plant interactions (Verpoorte and Memelink 2002). In fact, unlike the primary metabolites, such as chlorophyll, amino acids, nucleotides, carbohydrates, the secondary metabolites do not take part in fundamental processes such as metabolism.

The secondary metabolites diverge from these for their distribution in the plant kingdom. While primary metabolites they appear in all species of the plant kingdom, the secondary are only present in some plants families. For example, the glycosidic cardiokinetic are typical of the genus *Digitalis*.

Secondary metabolites can be classified on the basis of chemical structure (for example, having rings, containing a sugar), composition (containing nitrogen or not), their solubility in various solvents, or the pathway by which they are synthesized (e.g., phenylpropanoid, which produces tannins). A simple classification includes three main groups: the terpenes (made from mevalonic acid, composed almost entirely of carbon and hydrogen), phenolics (made from simple sugars, containing benzene rings, hydrogen, and oxygen), and nitrogen-containing compounds (extremely diverse, may also contain sulfur).

The study of plant secondary compounds can be considered to have started in 1806 when Friedrich Wilhelm Sertürner isolated morphine, "*principium somniferum"*, from opium poppy. This first demonstration that the active principle of a plant drug can be isolated and attributed to a single chemical compound initiated natural product chemistry. In rapid sequence the isolation of one active principle after another followed. The speed at which this field developed, greatly influenced and directed major areas of organic chemistry, particularly synthetic, analytical and pharmaceutical chemistry. It also advanced the first foundations of pharmaceutical industry and drug research. The first synthesis of a secondary product, indigo, by von Baeyer in 1886, provided a milestone in synthetic organic chemistry. The chemical structure of morphine was elucidated in 1923, whereas the first total synthesis of its complex structure was not completed until 1950, almost 150 years after its isolation. These few introductory remarks should just recall and emphasize the "prehistory" of plant secondary metabolism which started and stayed for a long time Natural Product Chemistry with all its facets. For about 150 years after the

isolation of morphine, the plant was almost exclusively used as a profitable and inexhaustible source for novel natural products. First experiments addressing biosynthetic mechanism started not before the early 1950s, when the first radioactively-labeled precursors became available.

In the 1950s, secondary metabolites were regarded as metabolic waste or detoxification products (Peach, 1950 and Reznik, 1960). This view changed in the 1970s with the increasing biochemical knowledge of secondary metabolism. Secondary metabolites were now no longer considered as inert end products but as dynamic components of plant metabolism (Barz and Köster, 1980). Typical attributes of plant secondary metabolism were: “expression of shunt and overflow metabolism”; “expression of plants’ luxurious metabolism”; “flotsam and jetsam on the metabolic beach”; “playground of biochemical evolution” (Zähner, 1979), (Mothes, 1980), (Haslam, 1986) and (Luckner, 1990). An ecological role was generally only accepted due to a coincidental quality of a secondary compound (e.g., toxicity and bitter taste). The only generally accepted feature of secondary metabolism was its emergence from primary metabolism.

Sites of synthesis and accumulation of metabolites

Secretion is defined as the passage of substances produced from within the cell outside of its plasma membrane. These substances perform a specific function in the organism in which they are produced. The secretion is usually the result of metabolism of all cells, but it is particularly evident in the glandular cells. The plant cells have the ability to extrude the substances secreted out of the protoplasm or by the accumulation in vacuoles, and this depends on the type of substances. Secretion can occur with intracellular accumulation, such as when you have the storage of many substances in the cell wall (lignin, cutine), with intracellular secretion of substances secreted in compartments surrounded by membranes inside the plasma membrane or secretion extracellular, through the release of substances secreted outside the plasma membrane (Fahn.,1988). The cells can also be divided according to the type of compound secreted, those which produce hydrophilic substances and those that produce lipophilic substances.

At the first group belong mucilaginous glands, digestive glands, idatodi, nectars etc. In the second group we have instead trichomes glandular, idioblasti, resin canals and lisigen pockets. The trichomes glandular protuberances are present in the epidermis of many plants species. The morphology of these structures is very different and are formed as a result of divisions anticlinals and periclinals. Regard to the structure they are composed of a basal cell, one or more cells that produce secreted and in many species there is a stem cell that connects the two cells. Between the various families that of *Labiateae* is rich in species that produce flavorings used for various purposes in the food industry and in the cosmetic industry (Doaige A.R.,1992).

In this family study of glandular trichomes has been thoroughly done, for example in genus belong to the subfamily *Nepetoidee* where there are two types of trichomes: peltata and capitati. The peltata trichomes have a secretory head consisting of 4-14 cells, supported by a stem cell that connects to the basal cell. Tricomi capitati in the single cell spreads secreted into the subcuticolare space without store, as happens in trichomes peltata (Doaige A.R.,1992). *Lavandula officinalis* in the inner part of the corolla has a particular type of tricomi consists of

a single secretory cell and numerous protuberances on the stem cells, while the outside of the corolla have trichomes with a completely different structure. In *Lavandula stricta* and *L. coronopifolia* have been described 13 different types of glandular trichomes (Werker E.,Ravid U., Putievsky E., 1985). In *Salvia officinalis* are three types of glandular trichomes, two capitati cells with long and short stem and one or two secretory cells, and peltata with a secretory head of 12 cells. Also in *Salvia sclarea* and *S. dominica* were observed wide variabilty in the form and mode of secretion of trichomes capitati both on vegetative organs (leaves and stems) and those in reproductive (Werker E.,Ravid U., Putievsky E., 1985). Another type of structures are secretory channels present in *Pinaceae*, *Compositae*, *Umbelliferae* etc. In the conifers secretory channels produce a resin formed by mono- sesqui- and diterpeni and therefore are known as resin canals. In these plants the resin canals are found throughout the body of the plant and are formed by elongated structures enveloped by epithelial cells surrounding an inner space. These cells are in turn surrounded by one or more layers of cells with thick cell walls and relatively rich in pectic substances. Channels resin best known are those of the genus Pinus which may occur in cross-sections of roots, stems and leaves (Werker E., Fahn A.,1969). Another type of internal secretory tissues there are the cavities or pockets lisigene present in many households (*Myrtaceae*, *Leguminosae* etc). In some species is observed the formation of meristemoide tissue characterized by cells with dense cytoplasm and a large nucleus. The first two divisions of this structure form two layers bicellulari, one upper and one lower. The latter are formed by secretory cells which will generate the epithelium surrounding the interior space.

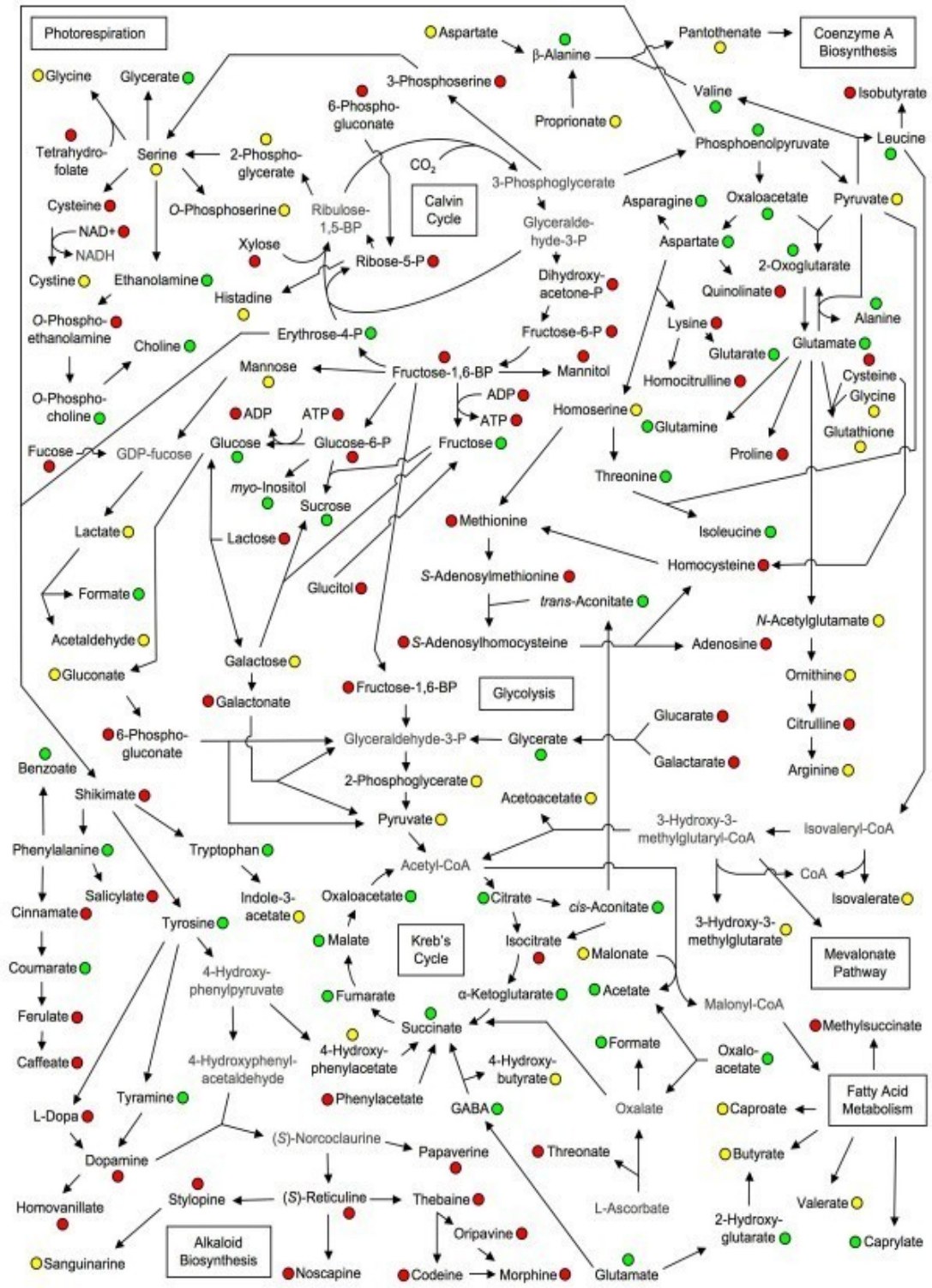


Fig. 2 Metabolite linkage map representing primary and secondary plant metabolism in opium poppy. The circles associated with each metabolite indicate whether the metabolite was detected (green), not detected (red) or masked (yellow). Data could not be obtained for metabolites shown in grey because information regarding their standard ^{1}H NMR spectra was not available (*Zulak et al 2008)*.

Extraction of metabolites

A very important part in the study of metabolites is the technique of extraction. Metabolite may accumulate in different concentrations in different parts of the plant and in some cases there may be other compounds in the same plant. Very important is also (in addition to the state of health of the plant), the type of solvent used. The ideal solvent for polar compounds is water, which is used in many cases in combination with other solvents less polar, for example alcohols. The lipids and other apolar molecules are predominantly soluble in non polar solvents, among which the most used are chloroform, petroleum ether and short chain hydrocarbons (pentane, hexane etc.). Whatever the type of compound the solvent must to extract molecules quickly from the tissues. To extract more easily the molecules are used to shatter of the tissues so that the solvent free the substances accumulate in the cells. In some cases, these operations make contact with the secondary metabolites with lithic or oxidative enzymes that attack molecules by altering the structure. Indeed in many cases, the extractions are carried out cold, because the low temperatures inhibit the activity of lithic enzymes, it is sometimes necessary to use liquid nitrogen which crystallizes the tissue making them fragile, but at the same time more easily extractable. Once extracted the molecules are separated from all other cellular components with the same degree of polarity using techniques based on preparative chromatography of various kinds. The main task of the chromatography is to separate the molecules and this is done by exploiting the different nature of the compounds contained in the mixtures extraction. The separation occurs because the molecules interact with the solvent, but also with the surface of the particles forming the stationary phase. Of the many chromatographic techniques are the most used ones in the liquid phase, but for certain molecules are equally effective techniques such as using mobile phase of gas. In liquid chromatography (on column, TLC, HPLC), the mixture of compounds is deposited on the stationary phase and an appropriate mixture of solvents will make the move into the mobile phase mixture and separate the molecules. In chromatography with the mobile gas phase there is always a stationary phase with which the molecules interact driven by a gas. Both these techniques have the capability to separate

individual compounds from complex mixtures, however, do not provide data on the molecular nature of the substance separated. Therefore, these two techniques are associated with mass spectrometry, a tool connected to the column of a liquid or gas chromatograph capable of fragmenting the separated substances generating positive ions. These ions are subjected to acceleration and travel specific paths, so you can calculate the ratio charge / mass. A detector intercepts these trajectories provide a fingerprint of the molecule and the molecular fragments can be traced back to the chemical structure of the molecule.
Other methods of extraction are:

Distillation: is a technique which consists of subjecting a liquid to boil and collect in a container other volatile liquids made by heating. By steam distillation are extracted, from complex mixtures of organic volatile, the essences. May be aware of water vapor, and in this case the drug is crossed by water vapor in order to extract the volatile components such as essential oils. This type of distillation can also be preceded by maceration. In this case the drug is made by macerating a solvent appropriate (which may be the alcohol) and then distilled.

Enfleurage: process of adsorption of volatile essences of flowers and drugs in fixed solid fats. The fat containing the essence is then treated with appropriate solvents to extract the pure essence. It is a technique used by the perfume industry. In this technique the fresh flowers are placed for about 24 hours on a glass plate coated with a thin layer of oil or grease, after which the flowers are removed and replaced with fresh. The fat must be odorless, does not rancid or deteriorate over time. With the enfleurage are obtained compounds of fragrant substances called "pommade", typical of the south of France. This technique is used for those drugs that are unable to provide sufficient quantities of essential oil when distilled or flowers that do not tolerate prolonged exposure to heat.

Digestion: is a type of maceration conducted at a temperature ranging from 40 to 60 °C. Is done in a closed flask and is used for substances of low solubility in cold and changes over 65 °C.

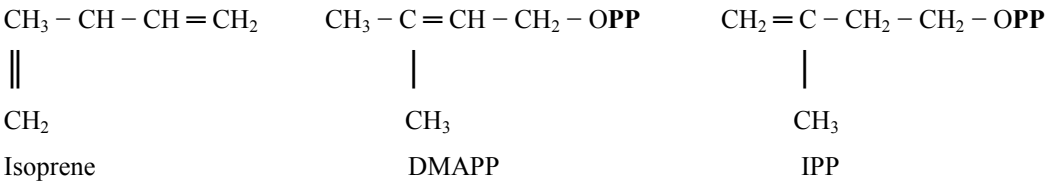
Percolation: extraction conducted at room temperature, where the solvent flowing slowly over drug dry properly prepared. These are tools of steel or glass in the shape of a vertical cylinder with a tap in the lower and in the upper there is a lid with a hole for passing the solvent. Above the taps, a pore is used to support the drug and acts, also, as a filter. Usually, a hydroalcoholic solvent is uses for extracting the active ingredients.

Classes of metabolites

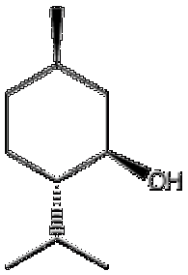
The secondary metabolites can be divided into three main classes:

- Terpenes
- Phenolic compounds
- Compounds containing nitrogen

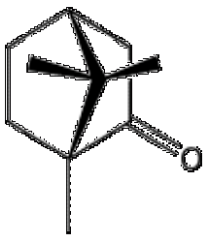
The terpenes are the largest class of secondary metabolites. They may be oil or may contain oxygen and alcohols, ketones and aldehydes. The terpenes which contain oxygen are called terpenoids. These natural substances, structurally different, are grouped together for their common origin biosynthetic from acetil CoA and their insolubility in water. The terpenes are formed from the combination of elements to five carbon atoms called isoprene units and are classified according to the number of these units in emiterpenis (C5), monoterpenes (C10), sesquiterpenis (C15), diterpenis (C20), sesterterpenis (C25), triterpenes (C30) and tetraterpenis (C40) (Bruice, 2005). The isoprene is the basic constituent units of these compounds, is produced naturally but is not involved in their biosynthesis. Isoprene units that are involved in the biosynthesis are esters dimetilallil diphosphate diphosphate (DMAPP) and isopentenil diphosphate (IPP) that are joined to form the various terpenes (Dewick, 2001)



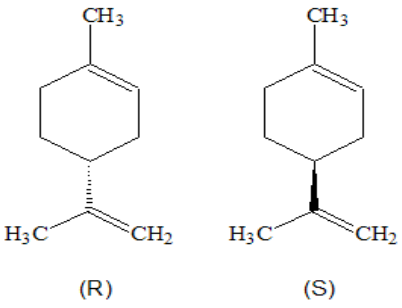
Many plants produce mixtures of volatile monoterpenes and sesquiterpenes, called essential oils, that impart a distinctive odor to the leaves that produce them and are used in fragrances and flavorings. A triterpenes, the Squalene, plays an important biological role as precursor of steroids. Carotenoids are tetraterpenes among these there is eg β-carotene, which is the compound that gives orange color to carrots and apricots (Bruice, 2005). The terpenes, in particular essential oils, play an important role in plant defense as deterrents toxins against a large number of insects and mammals pests. In the plant, these compounds can be found within glandular trichomes who extend outside the skin and serve as a warning to the herbivore toxicity of the plant. The essential oils are extracted through steam distillation and from a commercial point of view are important to flavor foods and for the perfume industry. The volatile terpenes as well as being a direct defense of the plant helps it to attract others insects to defend themselves. The conifers accumulate monoterpenes in the resin canals of needles, twigs and the trunk, following the attack of pathogens, such as bark beetles, the plant increases the production and accumulation of monoterpenes that are toxic to these predators (Hartmann, 2007) .



Menthol

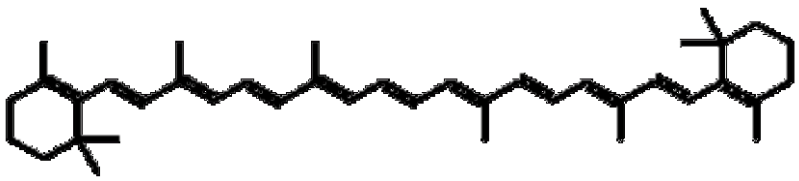


Camphor



(R) (S)

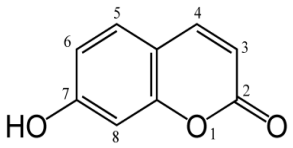
Limonene



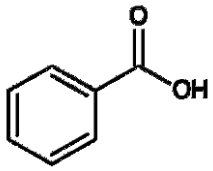
β-carotene

Fig. 3 Chemistry structure of same monoterpenis

The phenolic compounds are a heterogeneous class of chemical. These compounds are divided in different classes, from simple phenols such as benzoic and cinnamic acid, stilbenes and coumarin to those more complex like flavonoids and antocianine. Benzoic acids are the simplest molecules, consisting of a ring with a benzoic acid group in position 1. Substituents of different nature have been found, but the most common are -OH and -CH3 groups (Bruice, 2005). Cinnamic acid molecules are the basis for the construction of complex phenolic compounds. Flavonoids consist of three rings of which two are aromatic, and are found in all plants except for *Algae.* Phenols are important in fruit and vegetables, in which determine the color and flavor. In particular, phenolic acids are associated with the sour taste, to the astringent tannins, while the bitter taste is often associated with some flavonoids such as naringenina and neohesperidine. The color, finally, is determined by the presence of anthocyanins and their reactions co-pigmentation.

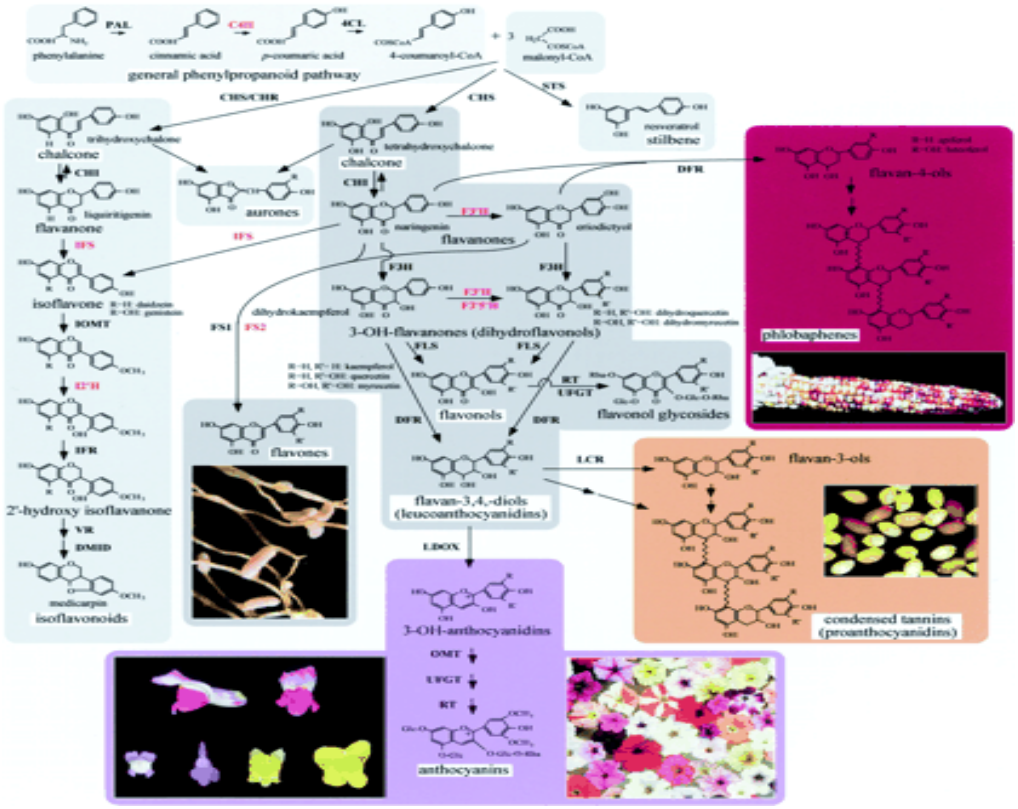


Umbelliferone



Benzoic acid

Fig. 4 Chemistry structure of some phenolic compounds



Plant Physiol, June 2001, Vol. 126, pp. 485-493 Brenda Winkel-Shirley

Fig. 5 Pattern of flavonoids biosynthesis

Many metabolites have one or more nitrogen atoms in their structure. Among these, the most important are alkaloids, cyanogenic glucosides, glucosinolate etc. Alkaloids are heterocyclic compounds for the nitrogen with potent pharmacological action. The glucosinolates are compounds which contain also a sulphur atom. Very important are cyanogenic glucosides, present in many plants used as food for humans and animals. When tissues of these plants are destroyed, they release hydrogen cyanide, and this causes many cases of poisoning cyanide. Depending on their precursor, amino acids are classified as aliphatic, aromatic or cyclopentenoid.

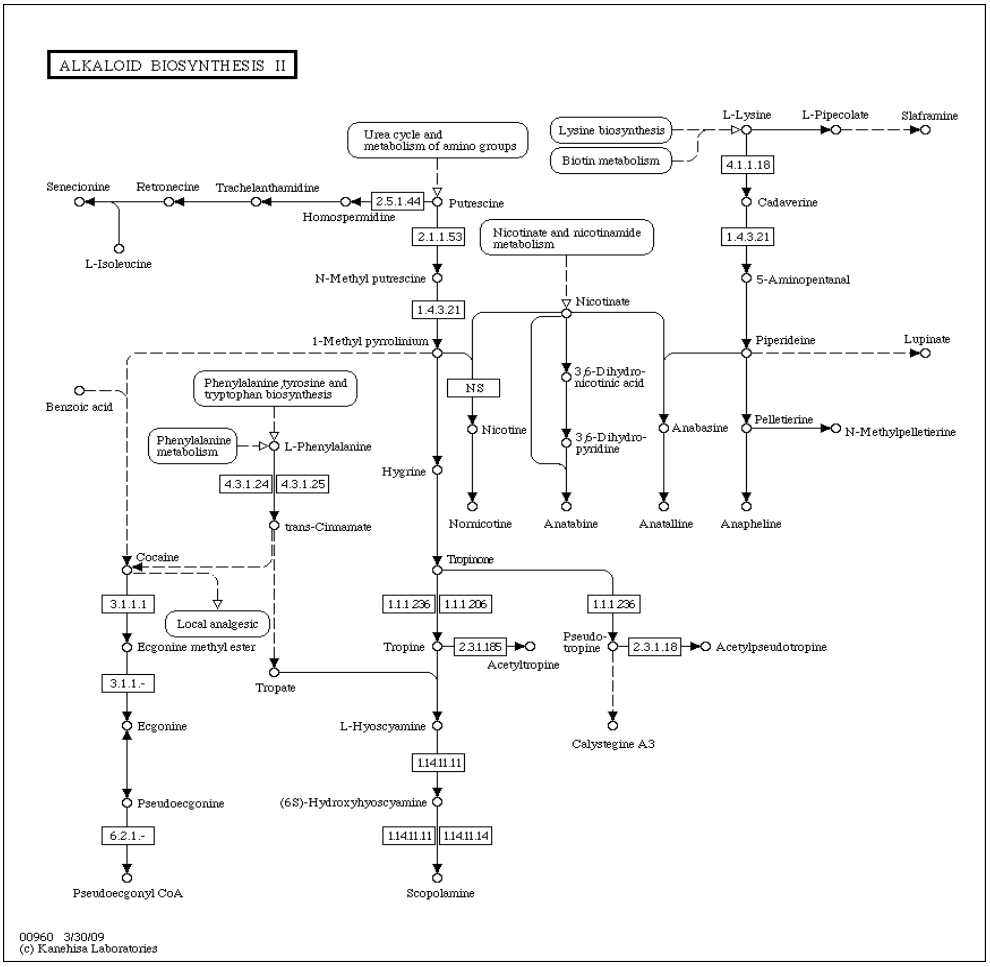


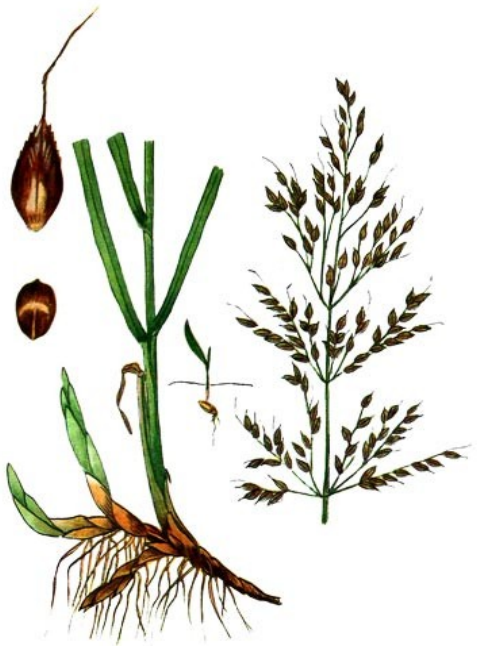
Fig. 6 Pattern of alcaloids biosynthesis

Sorghum bicolor (L.) Moench

Sorghum originated in the north-eastern quadrant of Africa, where the greatest variability in wild and cultivated species has been found to this day. It was probably domesticated in Ethiopia by selection from wild sorghum between 5,000 and 7,000 years ago. From the center of origin, it was distributed along trade and shipping routes throughout Africa, and the Middle East to India at least 3,000 years ago. It reached China along the silk route. Sorghum was first taken to the Americas through the slave trade from West Africa. It was reintroduced in late 19th century for commercial cultivation and has subsequently been introduced into South America and Australia.

Sorghum is now widely found the drier areas of Africa, Asia, the Americas and Australia.

Total annual production ranges from 40-45 million t from approximately 40 million ha, making sorghum the fifth most important cereal in the agricultural world, after wheat, rice, maize and barley. The most important producers are the United States with annual production of 17 million t of grain from 4 million ha, India (11 million t from 12.5 million ha), Nigeria (6 million t from 5.7 million ha) China (5.5 million t from 1.5 million ha), Mexico (4.5 million t from 1.3 million ha), and Sudan (3 million t from 5 million ha) (ICRISAT, 1999).



Sorghum is a vigorous grass that varies between 0.5-5.0 m in height. It is usually an annual. It produces one or many tillers, which emerge initially from the base and later from the stem nodes. The root system consists of fibrous adventitious roots that emerge from the lowest nodes of the stem, below and immediately above ground level. Roots are normally concentrated in the top 0.9 m of soil, but may extend twice that

depth and can extend to 1.5 m in lateral spread. The stem is solid and erect. Its center can be dry or juicy, insipid or sweet to taste. The center of the stem can become pithy with spaces. Leaves vary in number from 7-24, depending on the cultivar. They are borne alternately in two ranks. Leaf sheaths vary in length between 15-35 cm and encircle the stem with their margin overlapping. The leaf sheath often has a waxy bloom. Leaves are from 30-135 cm long and 1.5-3 cm wide, with a flat or wavy margins. Midribs are white or yellow in dry pithy cultivars or green in juicy cultivars. The flower is a panicle, usually erect, but sometimes recurved to form a goose neck. The panicle has a central rachis, with short or long primary, secondary and sometimes tertiary branches, which bear groups of spikelets. The length and closeness of the panicle branches determine panicle shape, which varies from densely packed conical or oval to spreading and lax. Grain is usually partially covered by glumes. The seed is rounded and bluntly pointed, from 4-8 mm in diameter and varying in size, shape and color with cultivar.

Sorghum is adapted to a wide range of environmental conditions but is particularly adapted to drought. It has a number of morphological and physiological characteristics that contribute to its adaptation to dry conditions, including an extensive root system, waxy bloom and leaves that reduces water loss, and the ability to stop growth in periods of drought and resume it again when conditions become favourable. It is also tolerant to waterlogging and can be grown in high rainfall areas. It is, however, primarily a crop of hot, semi-arid tropical environments with 400-600 mm rainfall that are too dry for maize. It is also widely grown in temperate regions and at altitudes of up to 2,300 m in the tropics.

Sorghum can be grown successfully on a wide range of soil types. It is well suited to heavy vertisols found commonly in the tropics, where its tolerance to waterlogging is often required, but is equally suited to light sandy soil. It tolerates a range of soil pH 5.0-8.5 and is more tolerant to salinity than maize. It is adapted to poor soils and can produce grain soils where many others crops would fail (ASA Technical Bulletin Vol. AN20-1999).

In agriculture of the Third World, the grain is used directly for human consumption, these countries could not afford the processing livestock, infact the

yields are very low, 0.5-1 t/ha, for both early cultivation technique and adverse environmental conditions. In advanced agriculture, the grain is intended to animal feed, in competition with corn, which has similar nutritional value. Moreover, a certain part is devoted to industrial transformation in ethyl alcohol.

The aptitude to produce cyanide or cyanogenesis has long been recognized in plants. At least 2,650 species from more than 550 genera and 130 families possess the capacity to make cyanogenic glycosides (Hegnauer 1986; Seigler 1991). Cyanogenic plant species include ferns, gymnosperms, and both monocotyledonous and dicotyledonous angiosperms.

Although cyanogenesis is a widespread phenomenon, the actual cyanogens have been isolated and studied from only 475 species of plants (Seigler 1991). From these studies, the natural occurrence of about 60 different cyanogenic compounds has been established (Nahrstedt 1987a; Seigler 1991).

Plants containing cyanogenic glycosides, usually also contain β-glycosidases, capable of degrading the cyanogenic glycoside to a cyanohydrin (aglycone) and a sugar (Eksittikul and Chulavatnatol 1988; Hughes 1993; Kuroki and Poulton 1987; Mkpong *et al*. 1990). In many instances, a second type of enzyme (hydroxynitrile lyase) catalyzes the dissociation of the cyanohydrin to a carbonyl compound and hydrogen cyanide (Hughes *et al*. 1992, 1994; McMahon *et al*. 1995; Poulton 1990; Selmar *et al*. 1989).

Normally, the substrate and enzymes are compartmentalized within the plant, and cyanide release does not occur unless the plant is damaged (Conn 1981, 1993; Poulton 1988; Poulton and Li 1994; Selmar 1993).

Cyanogenic glucosides are also present in food and forage plants, such as cassava, sorghum and almonds. Insufficient removal of the cyanogenic glucosides present in cassava roots constitutes a potential health hazard for the millions of people in Africa, South-East Asia and South America, who are dependent on these roots as their major staple food (Davis 1991; Koch *et al*. 1995b; Nahrstedt 1993; Rosling 1988; Spencer 1994; Tylleskär *et al*. 1992; Wilson 1987).

The expression of cyanogenesis can be influenced by stress and other environmental factors (Conn 1979b; Jones 1988; Seigler 1991). For example the content of cyanogenic glycosides in sorghum varies greatly during the growing season and is especially high when the plants undergo frost or drought (Boyd *et al*. 1938; Willaman and West 1916). Nonetheless, the distribution of cyanogenic glycosides is of systematic interest: certain structural types of cyanogenic glycosides are associated with specific groups of plants (Hegnauer 1986; Saupe 1981).

The presence of cyanogenic glucosides is often thought to make cyanogenic plants less prone to attack by microorganisms, as well as by other pests and herbivores (Bellotti and Riis 1994; Compton and Jones 1985; Conn 1979b; Georgiadas and McNaughton 1988; Jones 1988; Nahrstedt 1985), but the quantitative importance of cyanogenic glucosides to deterrence of generalist feeders is difficult to assess (Hruska 1988).

Most animals are able to metabolize small quantities of cyanide, and many specialized herbivores exist that are capable of dealing with much larger quantities (Conn 1979b; Seigler 1991).

Cyanide released from field-grown sorghum is an effective plant defense and probably accounts for most of this crop's lack of palatibility to acridids in West Africa and India (Bernays 1983). The effectiveness of cyanide in many plants appears to be related to the release rates during chewing and not to the levels of cyanogenic glycosides present (Bernays 1983).

Most of the compounds are derived from five amino acids, others from the nonprotein amino acid 2-(2-cyclopentenyl)glycine, and one probably from nicotinic acid (Seigler, 1991).

Cyanogenic glycosides derived from tyrosine:

> the best-known of this series is dhurrin, which make up to 30% of the dry weight of the leaves and coleoptiles of etiolated sorghum seedlings (Halkier and Møller 1989; Saunders and Conn 1977).

Cyanogenic glycosides derived from phenylalanine:

> the best-known is amygdalin, which is widespread in seeds of members of the Rosaceae, such as almonds, apples, peaches, cherries and apricots.

Meta-substituted cyanogenic glycosides:

although the biosynthetic origin has not been definitely established for all meta-substituted cyanogens, (S).zierin, is derived from phenylalanine (Nahrstedt 1992; Nahrstedt and Schwind 1992).

Cyanogenic glycosides derived from valine and isoleucin:

the best-known are linamarin, (R)-lotaustralin, (S)-epilotaustralin, linustatin, neolinustatin and sarmentosin epoxide.

Cyanogenic glycosides derived from leucine:

in this group are know (R)-epiproacacipetalin, (S)-proacacipetalin, (S)-proacaciberin, (S)-heterodendrin, (R)-epihetetherodendrin, 3-hydroxyheterodendrin and (S)-cardiospermin.

Cyanolipids:

the α-hydroxynitrile of a leucine-derived cyanohydrin is esterified with a long-chain fatty acid (C_{18} or C_{20}). In the seed olis of several species of the family *Sapindaceae* produce cyanogenic lipids (Mikolajczak 1977; Seigler 1991).

Cyanogenic glycosides with a cyclopentenoid ring structure:

several cyanogens that contain a cyclopentenoid ring structure (or related structure) appear to be derived from 2-(2-cyclopentenyl)glycine, a nonprotein amino acid (Cramer and Spener 1976; Jaroszewski and Olafsdòttir 1986; Jaroszewski *et al*. 1987a; Nahrstedt 1987a).

Acalyphin:

the cyanophoric compound acalyphin from *Acalypha indica* (*Euphorbiaceae*) appears to be derived from nicotinic acid metabolism (Nahrstedt 1987a).

Nitroglycosides:

Nitro acids, nitro alcohols, and their glycosides have been reported from plants in several plant families (Seigler 1991).

Nitrile glucosides:

another series of noncyanogenic nitrile glycosides are found in members of the *Simmondsiaceae*, *Aquifoliaceae*, *Menispermaceae*, *Fabaceae*, *Boraginaceae*, *Ranuncolaceae* and *Crassulaceae* (Seigler 1991).

The biosynthesis of dhurrin and enzymes involved in Sorghum bicolor

Sorghum synthesizes the cyanogenic glucoside dhurrin (β-D-glucopyranosyloxy-(S)-hydroxymandelonitrile), which is derived from the amino acid L-tyrosine. The biosynthesis of dhurrin involves two multifunctional cytochromes P-450s (CYP79A1 and CYP71E1) (Halkier and Møller, 1991; Kahn *et al*., 1997; Koch *et al.*, 1992; Sibbesen *et al*., 1994; Sibbesen *et al.*, 1995) and a UDP-glucose glycosyltransferase (UGT85B1) (Hansen *et al*., 2003; Jones *et al*., 1999).

Biosynthetic studies with radioactively labelled precursors and trapping experiments, in which unlabeled putative intermediates were included in the microsomal reaction mixtures, identified N-hydroxyamino acids, N,N-dihydroxyamino acids, (E)-aldoximes, (Z)-aldoximes, nitriles, and cyanohydrins as key intermediates in the biosynthetic pathway. All the compounds, except the N,N-dihydroxyamino acids, have been chemically synthesized and are metabolized by the microsomal system (Møller and Conn 1978; Shimada and Conn 1977; Halkier and Møller 1990; Halkier *et al*. 1989). Cytochrome P450s are heme-etiolated monooxygenases, which consist of a NADPH-cytochrome P450 oxidoreductase and a cytochrome P450. The NADPH-cytochrome P450 oxidoreductase uses NADPH to produce electrons for the redox-reaction catalysed by the cytochrome P450, which is the substrate-binding and catalytic part of the monoxygenase (Halkier, 1996). Microsomal activity is dependent on the presence of oxygen and NADPH. Stoichiometric measurements of oxygen consumption demonstrated that conversion of the parent amino acid tyrosine to the corresponding cyanohydrin p-hydroxymandelonitrile, proceeds with the consumption of three molecules of oxygen, indicating the involvement of three hydroxylation reactions. Two molecules of oxygen are consumed in the conversion of the amino acid to the aldoxime, whereas a single oxygen molecule is consumed in the conversion of the aldoxime to the cyanohydrin (Halkier and Møller 1990). UDP-glucose glycosyltransferases (UGTs) are characterized by the usage of UDP activated sugar molecules. In general the UGTs convert toxic or unstable aglycons to stable and non-reactive compounds, which can be stored within the plant cell or

to facilitate a higher degree of solubility to aglycons. It is idea that the plant UGT family has co-evolved with the cytochrome P450 family to hinder the accumulation of toxic products produced by the cytochrome P450. The evolution of both enzyme families are therefore thought to be a result of the new harsh conditions the terrestrial plants had to overcome for the colonization of land (Paquette *et al*., 2003).

CYP79A1 is responsible for the conversion of L-tyrosine to the (Z)-p-hydroxyphenylacetaldoxime (Halkier *et al*., 1995; Koch *et al*., 1995; Sibbesen *et al*., 1994; Sibbesen *et al.*, 1995), which is further converted into p-hydroxymandelonitrile by CYP71E1 (Kahn *et al*., 1997; Bak *et al*., 1998). The latter product is glycosylated by UGT85B1 to produce dhurrin (Jones *et al.*, 1999; Thorsøe *et al*., 2005). CYP79A1 is highly substrate specific and takes merely L-tyrosine as its substrate, whereas CYP71E1 converts aromatic oximes more efficiently than aliphatic oximes and has therefore less stringent substrate requirements (Kahn *et al*., 1999).

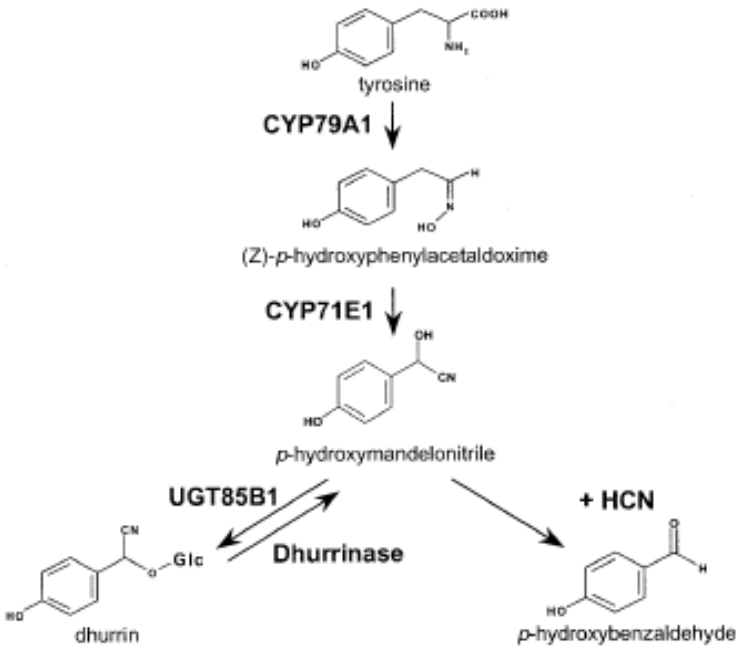


Fig. 8 The biosynthetic pathway for the cyanogenic glucoside dhurrin in Sorghum.

Metabolic channelling in the biosynthesis of dhurrin

The biosynthetic pathway of cyanogenic glucosides is highly channelled (Møller and Conn, 1980; Halkier *et al*., 1989; Kahn *et al*., 1997; Kristensen *et al*., 2005; Sibbesen *et al.*, 1995) and therefore thought to form a metabolon (Jørgensen *et al*., 2005; Nielsen *et al*., 2008), which is a multi-enzyme complex that allows channelling of toxic unstable intermediates. The metabolon involved in the biosynthesis of dhurrin consist of the two membrane-bound cytochrome P450s (CYP79A1 and CYP71E1) and membrane-bound cytochrome P450 reductase. The soluble UGT85B1 is thought to be in tight interaction with the membrane –bound enzymes. This allows an immediate conversion of the toxic intermediate because the active sites of CYP79A1 and CYP71E1 are closely connected with each other.

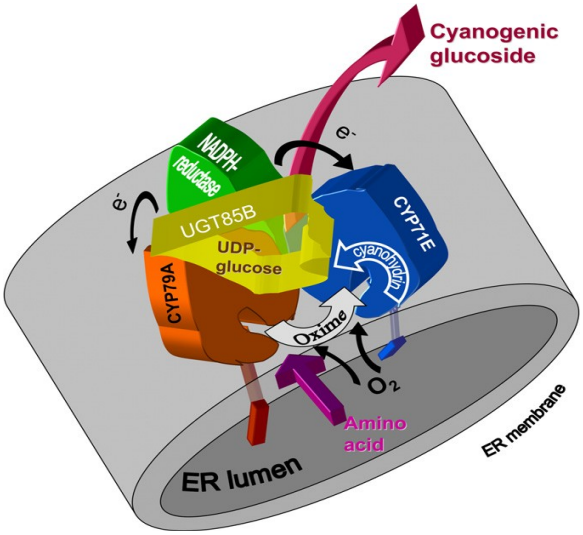


Fig. 9 . Metabolon composed of the two membrane bound cytochromes P450 CYP79A1 and CYP71E1 and the cytosolic UGT85B1 localized at the cytosolic surface of the endoplasmic reticulum. A NADPH dependent membrane bound NADPH-cytochrome P450 oxidoreductase provides reducing power in the form of single electrons. The substrate L-tyrosine enters the catalytic site of CYP79A1 together with molecular oxygen O2. Two molecules of O2 are used by CYP79A1 to form Z-phydroxyphenylacetaldoxime. CYP71E1 converts Z-p-hydroxyphenylacetaldoxime to the cyanohydrin p-hydroxymandelonitrile with the consumption of a single molecule of oxygen. The aglycon is glycosylated by the UDPG dependent glycosyltransferase, UGT85B1, and released as dhurrin.

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This would also be the reason for the difficulties in isolation of intermediates (Jørgensen *et al*., 2005; Kristensen *et al*., 2005; Winkel, 2004; Nielsen *et al*., 2008). Jones *et al*. (1999) found that UGT85B1 is inhibited by p-

hydroxybenzaldehyde. This compound is the degradation product of the substrate (p-hydroxymandelonitrile) of the glucosyltransferase. Metabolic channelling could hinder this inhibition, because p-hydroxymandelonitrile is not released into the cytosol and thereby not able to be converted into p-hydroxybenzaldehyde.
Recently the study by Nielsen *et al*. (2008) have investigated the possibility of the biosynthesis of dhurrin being situated in a metabolon by labelling the enzymes with fluorescent proteins CFP or YFP and transiently express them in *Arabidopsis thaliana* and *Sorghum bicolor*. They found using Confocal Laser Scanning Microscopy (CLSM) that the three enzymes involved in dhurrin synthesis are close connected inside the cells, which supports the metabolon theory.

Catabolism of cyanogenic glucosides in Sorghum bicolor

Dhurrin is a cyanogenic glucoside, which is a plant defense compound and classified as phytoanticipines. The cyanogenic glucosides are stored in the plant vacuole and upon the tissue damage the CNG is mixed which the hydrolysing enzymes β-glucosidase and α-hydroxynitril lyases. This result in cyanogenesis, which releases toxic hydrogen cyanide also called the "cyanide bomb"(Kahn *et al*., 1999; Zagrobelny *et al.*, 2004). Cyanide is a toxic substance, mainly due to its affinity for the terminal cytochrome oxidase in the mitochondrial respiratory pathway (Brattsten *et al*., 1983). The lethal dose of cyanide for vertebrates lies in the range of 35-150 μmoli/Kg, if applied in a single dose. Much higher amounts of HCN can be tolerated if consumed or administered over a longer period, e.g. by the grazing of livestock (Davis and Nahrstedt, 1985).

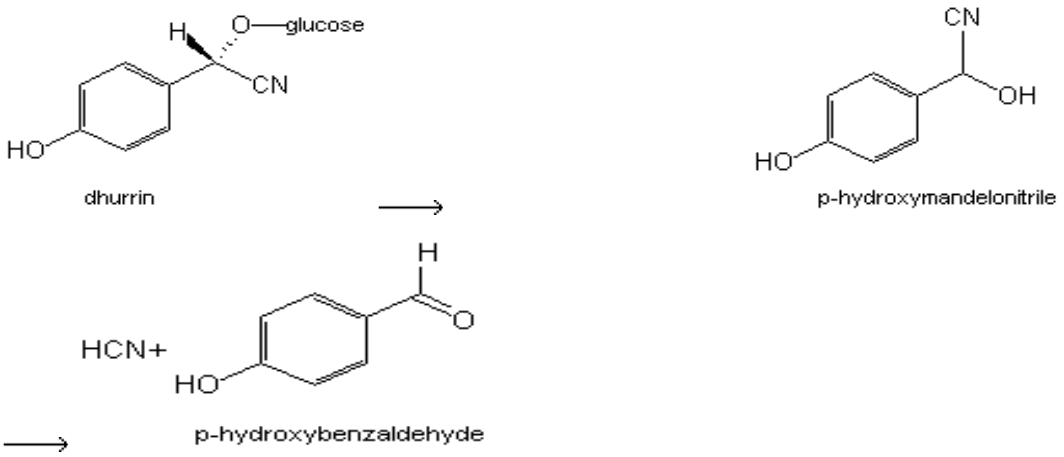


Fig. 10 Catabolism of dhurrin

Detoxification of cyanide in higher plants

Cyanide is produced, as a by-product in the biosynthesis of the plant hormone ethylene in all higher plants (Peiser *et al*., 1984) (Pirrung M.C., 1985) and therefore higher plants needs to detoxity them. In addition to this the cyanogenic plants are able to utilize this detoxification pathway when cyanogenic glucosides are degraded into cyanide. Cyanide, released from a cyanogenic glycoside in food by ß-glucosidase either of plant or from gut microflora origin and taken up (Conn, 1979a, 1979b; Oke, 1979, 1980; Nartey, 1980; Rosling, 1987; Gonzales & Sabatini, 1989), follows the known cyanide metabolic pathway and toxicokinetics both for animals and man. Cyanide is detoxified by the enzyme rhodanese, forming thiocyanate, which is excreted by urine (Conn, 1979a, 1979b; Oke, 1979, 1980). Due to several factors influencing hydrolysis of cyanogenic glycosides and the confounding influence of nutritional status (such as riboflavin, vit. B12, sodium, methionine intake) human case studies and epidemiological studies of the chronic toxicological effects have shown very variable results and were not conclusive. In addition, the data in these studies are rarely of a quantitative character (Conn, 1979a, 1979b; Oke, 1979, 1980; Nartey, 1980; Rosling, 1987). In several studies both in animals and man the toxicity of cyanogenic glycosides is often expressed as mg releasable cyanide.

In Sorghum there are three NIT4 isoforms SbNIT4A, SbNIT4B1 and SbNTI4B2, which in heteromeric complexes SbNIT4A/B1 SbNIT4A/B2 hydrolyses β-cyanoalanine (Jenrich *et al*., 2007). β-cyanoalanine is the intermediate in the detoxification pathway of cyanide in higher plants.

When cyanogenic plants are eaten slowly or over a period of time there may be no symptoms of cyanide poisoning. For example sheep can consume 15-20 mg/Kg per day when ingestion is distributed over the whole day (Coop, I.E. and Blakely, R.L.,1949). Human can consume 30-35 mg HCN per day from cassava in the form of gari (Uwakwe, A.A., Monanu, M.O. and Anosike, E.O. 1991). This is approximately 50% of the acute lethal dose for a 70 Kg human. Thus when doses are relatively small, mammals are capable of detoxifying HCN.

Toxicity on herbivores

Herbivory is a widespread feeding habit of terrestrial vertebrates. Plant material is generally a diffuse source of nutrients, and large amounts of material need to be ingested to maintain the metabolism of herbivorous mammals (Iason and Van Wieren, 1999). As a result, these mammals can consume a high proportion of plant biomass production and hence have an important influence on ecosystem function (Hester *et al*.,2006).

Because of plants immobile nature and vulnerability to herbivory, strong selection pressure exists to evolve antiherbivory adaptations (Rhoades, 1979). Antiherbivory strategies diverge along the lines of the major division within the plant kingdom. Monocotyledonous plants (e.g., grasses and sedges) tolerate herbivory; their basal meristems reduce the impact of grazing on plant survival by facilitating regrowth (McNaughton, 1983). Dicotyledonous plants tend to have chemical and physical defenses that discourage herbivory (Bryant *et al*., 1991). Chemical defense is manifest in the evolution of a large array of phytochemicals that appear to have minimal function in the primary metabolism of plants (Rosenthal and Berenbaum, 1991). These so-called plant secondary metabolites (PSMs) include alkaloides, terpenes, phenolics, glucosinolates, and cyanogenic glycosides. Mammalian

herbivores that ingest PSMs are liable to suffer a variety of adverse effects including reductions in nutrient availability and toxicity.

- *Gastrointestinal Effects*

Some PSMs remain in the gut where they may have local adverse effects. Condensed tannins, for example, are large polar molecules that cannot cross the gastrointestinal epithelium and are, therefore, not absorbed (Terrill *et al*., 1994). Tannins have an astringent taste and can bind and reduce the absorption of dietary proteins. Formyl phloroglucinols (e.g., jensenone, sideroxylonal) are aldehydes that react so rapidly with amine groups on proteins and other cellular constituents that they are not absorbed from the gut (McLean *et al*., 2004). However, these reactions result in adverse effects, mediated through serotonin release, which deter herbivory (Lawler *et al*., 1998; Wiggins *et al*., 2005).

- *Systemic Effects*

Many PSMs are lipophilic in character and can pass readily through the cell membranes of the gastrointestinal epithelium and be absorbed into the bloodstream, in which they will be delivered around the body to produce effects at distant sites.

For example, morphine and digoxin stimulate chemoreceptors in the area postrema of the brainstem, resulting in nausea (Saito *et al*., 2003). In some cases, vomiting ensues leading to removal of unabsorbed toxin. Emetic agents can also act in the gut, via the release of serotonin and substance P from enterochromaffin cells, exciting sensory neurons that project to the area postrema and others parts of the brainstem (Saito *et al*., 2003). Stimulation of the emetic center is also associated with the development of conditioned flavor aversions that deter herbivores from repeated ingestion of toxic plants (Zahorik *et al*., 1990). PSMs can target other organs with specific toxicity; for example, digoxin causes cardiac arrhythmias, pyrrolizidine alkaloids produce multiple organ toxicity and cancers (Fu *et al*., 2004), and terpenes cause depression of the central nervous system (Falk *et al*., 1991; Tibballs, 1995). PSMs can also target particular systems, such as cholinergic

neurotrasmission that is blocked by atropine (which occupies the muscarinic receptors of acetylcholine) and enhanced by physostigmine (which inhibits acetylcholinesterase). Sublethal toxicity can deter future feeding through learned aversions (Provenza, 1995) and taste associations (Pass and Foley, 2000).

- Pharmacodynamics: How effect depends on concentration of drug

The effect of a drug generally depends on the size of the dose taken and the consequent concentration produced at site(s) of action in the body (Wilkinson, 2001). The relationship between the log concentration of drug and its effect is sigmoidal rising to an asymptote (Ross and Kenakin, 2001). Note that there is a dynamic range where the effect is sensitive to changes in concentration; a characteristic maximum effect (E_{max}); and a low concentrations the effects are minimal or zero. We hypothesize that herbivore metabolic adaptations and ingestive behavior are directed toward preventing the concentration of PSMs in the body from exceeding tolerable levels.

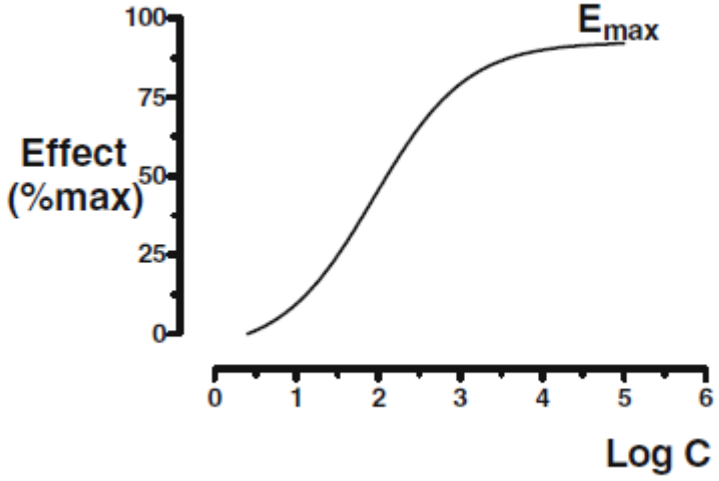


Fig. 11 General shape of the log concentration–effect curve

- Potency

Drugs and other PSMs differ markedly in their potency, measured by the amount (or concentration) required to produce a particular effect. For example, digoxin and atropine act therapeutically in humans at submilligram doses, whereas the

doses of salicin and quinine are 1,000 times greater. Among antifeedant PSMs, terpenes and phenolics are present at high concentrations in Eucalyptus leaf: up to 12% and 40% dry matter, respectively (Cork and Sanson, 1991), but the major antifeedant effect against marsupial folivores is exerted by formylphloroglucinols, which are present at significantly lower concentrations: mostly below 4% dry matter (Moore *et al*., 2004; Moore and Foley, 2005).

- *Tolerance*

Continued exposure to a drug can lead to tolerance: a reduced effect that is usually due to an adaptation by cells to the presence of the drug. This happens especially with drugs that act on the central nervous system, which for example becomes less sensitive to alcohol and nicotine in regular drinkers and smokers, respectively (Le Houezec, 1998; Nutt, 1999). Tolerance to alcohol can develop to such an extent that individuals can survive normally lethal blood levels (Davis and Lipson, 1986), as well as tolerance to opioids in that addicts may take 50 times the normal dose, an amount which could be lethal for an opioid-naïve person (O'Brien, 2001). However, not all drugs elicit tolerance, as for example the doses of digoxin, warfarin, and atropine-like drugs that do not need to be increased with their continued therapeutic usage (Hardman *et al*., 2001).

- *Removal of plant secondary metabolites*

It is self-evident that herbivores must eliminate ingested PSMs from their body or risk their progressive accumulation to harmful level. Metabolism and excretion are the processes used to eliminate PSMs from the body. Because the terrestrial kidney conserves water by extensively reabsorbing it from the renal tubules, lipophilic substances that can freely permeate the tubular epithelium are also extensively reabsorbed (Wilkinson, 2001). Thus, the more lipophilic a PSM, the more readily it will be absorbed from the gut and the less efficiently it will be excreted by the kidney. Animals possess a battery of enzymes that can trasform lipophilic

xenobiotics into water-soluble metabolites that are more readily excreted by the kidney.

Toxicity on humans

The cyanide ion (CN_) is the toxic moiety in hydrogen cyanide. The toxicity of simple cyanide salts, such as potassium and sodium cyanide is, therefore, similar to that of hydrogen cyanide (World Health Organisation –WHO, 2004).

The small size and moderate lipid solubility of hydrogen cyanide favours rapid absorption across mucous membranes and uptake by the alveolar epithelium before distribution throughout the whole body (Agency for Toxic Substances and Disease Registry-ATSDR, 2004). Absorption of hydrogen cyanide from the stomach is also rapid, with the acid environment favouring the non-ionized form and facilitating absorption (ATSDR, 2004).

Cyanide absorbed from the gastrointestinal tract undergoes first pass metabolism in the liver (WHO, 2004).

Ingestion of simple cyanide salts rapidly results in formation of hydrogen cyanide in the stomach as free cyanide ion is bound to hydrogen ion in the acidic stomach environment (WHO, 2004).

Cyanide in solution is absorbed across intact skin, due to its lipid solubility (WHO, 2004). Cyanide ion toxicity is mediated primarily by its high affinity for the ferric moiety of cytochrome c oxidase in mitochondria, a key component in oxidative respiration. This stable but reversible interaction blocks the last stage in the electron transfer chain, resulting in cellular hypoxia and a shift of aerobic to anaerobic cellular respiration, leading to cellular ATP depletion, lactic acidosis and cell and tissue death (WHO, 2004; Lindsay *et al*., 2004).

Metabolism of cyanide ion primarily involves its conversion to soluble thiocyanate (SCN-) by the enzyme rhodanese, with about 80% of cyanide detoxified by this route (WHO, 2004). This requires sulphane-sulphur as a co-factor i.e. one sulphur atom bonded to another sulphur atom such as in a thiosulphate salt (e.g. sodium thiosulphate). This conversion is irreversible and the thiocyanate ion may then be

readily excreted in the urine. Cyanide is therefore unlikely to accumulate in human tissues after chronic oral exposure (ATSDR, 2004).

Cyanide may also be metabolised by lesser pathways including the complexing of cyanide with cobalt in hydroxocobalamin to form cyanocobalamin (vitamin B12). Both these pathways have been exploited in antidote therapies for cyanide poisoning (Megarbane *et al.*, 2003).

While the majority of absorbed cyanide is excreted in the urine as thiocyanate, small amounts of free cyanide may also be excreted via the lungs, sweat, urine and saliva (WHO, 2004). A plasma half life of 20 min to 1 hour has been estimated for cyanides in humans (ATSDR, 2004).

The clinical signs are well described (Montgomery, 1969; Gosselin *et al.,* 1976) and include headache, dizziness, mental confusion, stupor, cyanosis with twitching and convulsions, followed by terminal coma (Conn, 1979a). The acute oral lethal dose of HCN for man was reported to be 60 mg (Sinclair & Jeliffe, 1961). For man the acute oral dose of HCN is usually given as 50-90 mg and for potassium cyanide as 200 mg, corresponding to 81 and 110 mg HCN respectively (Lehman, 1959). Data on the oral lethal dose of cyanide for man in four cases of suicide, calculated from the amount of HCN absorbed in the body at the time of death, and from the amount of HCN found in the digestive tract, differed considerably [calculated as mg HCN: 1450 (62.5 kg bw), 556.5 (74.5 kg), 296.7 (50.7 kg) and 29.8 (51 kg)] (Geitler & Baine,1938). This corresponds to doses varying from 0.58-22 mg/kg bw (WHO, 1965).

Other functions of dhurrin

There have been indications of cyanogenic glucosides to have another purpose for the plant than merely a defence compound. One of these other functions could be that cyanogenic glucosides are used as nitrogen storage (Selmar *et al*., 1988). It has recently been shown indications of SbNIT4A/B2 play a role in the turnover of dhurrin through the intermediate 4-hydroxyphenylacetonitrile. This means that dhurrin is metabolised without the release of toxic cyanide. If there is such a pathway present in cyanogenic plants it would enable the plants to reuse the

nitrogen channelled into cyanogenic glucosides to their primary metabolism. This could be the reason why the dhurrin content in etiolated Sorghum seedlings decreases after 3 days. It was however not been possible to decret 4-hydroxyphenilacetic acid, which is product of SbNIT4A/B2 hydrolyses of 4-hydroxyphenylacetonitrile. The enzyme or enzymes, which convert dhurrin into 4-hydroxyphenylacetonitrile is yet to be found but parallels to the epithiospecifier protein in the glucosinolate hydrolysis in the *Brassicaceae* could be in question (Jenrich *et al*., 2007). When the epitiospecifier protein in Arabidopsis is present upon hydrolysis of the glucosinolates by the myrosinase, the product is epithionitriles or nitriles instead of isocyanates, which is the active defence compound (Lambrix *et al*., 2001).

- *Sorgoleone*

Several Sorghum species, including *Sorghum halepense*, *S.vulgare*, *S.sudanense* and *S.bicolor* have been shown to cause allelopathic interference (Breazeale, 1924; Abdul-Wahab and Rice, 1967; Hussain and Gadoon, 1981; Putnam and DeFrank, 1983; Putnam *et al*., 1983; Alsaadawi *et al.*,1986). Both, autotoxicity and interspecific allelopathic effects, have been noted in sorghums (Rice, 1984). They produce and release cyanogenic glycosides and an array of phenolic acids that can contribute to suppression of plant growth (Martin *et al*.,1938; Guenzi and McCalla, 1966; Nicollier *et al.,*1983; Lehle and Putnam, 1983; Rice, 1984; Weston *et al*.,1989).

Sorghum roots exude biologically–active hydrophobic substances, one major component of which is known as sorgoleone, characterized as 2-hydroxy-5-methoxy-3-[(8'Z,11'Z)-8',11',14',-pentadecatriene]-p-benzoquinone (Chang *et al*., 1986; Netzly and Butler, 1986; Nimbal *et al*., 1996). The term sorgoleone is also used to describe a group of lipophilic p-benzoquinones structurally related to sorgoleone that are also produced by sorghum roots having a hydroxyl and a methoxy substitution at positions 2 and 5, respectively, and either a 15- or 17-carbon aliphatic tail with various degrees of unsaturation at position 3 (Netzlyc *et al.*, 1988). Sorgoleone is toxic to both small-seeded broadleaf and grass weeds at

concentrations as low as 10 μM in root uptake studies (Einhelling and Souza, 1992; Nimbal *et al.*, 1996). Work in Michigan demonstrated that *Sorghum bicolor* (grain sorghum) could be used as an allelopahtic cover crop to control weeds in vegetable crops and orchards (Putnam and DeFrank, 1983; Putnam *et al*., 1983). The primary mechanism of phytotoxic action of sorgoleone is associated with inhibiton of photosynthesis in higher plant system by competing for the binding site of plastoquinone on photosystem II, (Einhelling *et al*.,1993; Gonzales *et al*., 1997; Rimando *et al*., 1998). This lipophilic p-benzoquinone is also known to hinder electron transfer reactions involved in mitochondrial respiration and to inhibit the enzyme p-hydroxyphenylpyruvate dioxygenase (Rasmussen *et al*., 1997; Einhellig, 1995; Czarnota, 2001).

Recent work suggested that sorgoleone is produced only in living root hair (Czarnota *et al*., 2001). Root hair are tubular exstensions of epidermal cells that have their origin either in any protoderm cells or in specialized protoderm cells called trichoblast (Cormack, 1949; Row and Reeder, 1957; Ridge, 1995; Peterson and Farquar, 1996; Pemberton *et al*., 2001; Ryan *et al*., 2001). Root hairs contribute as much as 77% to the total root surface area of cultivated crops, forming the major point of contact between the plant and the rizosphere (Parker *et al.*, 2000). Root hairs play important roles in plant life processes, including uptake of water and nutrients, production of substances that mediate plant-microbial associations, regulation of plant growth, and determination of microbial community structure in the plant rizosphere (Fan *et al*., 2001; Grierson *et al*., 2001; Michael, 2001; Inderjit and Weston, 2003).

ASSUMPTIONS

On the basis of previous experiments, it is known that the synthesis of dhurrin is located in the stem and this at the beginning was considered as a single complex, but it is composed of many overlapping sheaths. This means that the shown activity of the biosynthetic enzymes by Busk and Møller (2002), might be situated in a specific sheath within the overlapping sheaths.
In this project it has been assumed that the biosynthesis of dhurrin takes place in a metabolon and therefore that the metabolon consist of one of each of the biosynthetic enzymes, CYP79A1, CYP71E1 and UGT85B1.

The location of the biosynthesis of dhurrin will be investigated by microsomal preparations, its will indicate the activity of the dhurrin metabolon and the tissue in which the synthesis takes place. It is known that microsomes can be efficiently prepared from 3-day etiolated Sorghum seedlings (Halkier and Møller, 1989). From this we also know that the last enzyme in the pathway is lost during the preparation of microsomes so the end product will be seen as p-hydroxybenzaldehyde.
This product is seen due to the degradation of the unstable intermediate p-hydroxymandelonitrile to p-hydroxybenzaldehyde. The presence of p-hydroxybenzaldehyde thereby indicates the activity of the metabolon.
The location of the dhurrin metabolon will be examined by labelling the mRNA encoding CYP79A1 instead of labelling all the involved enzymes mRNAs, whereas the enzymes (β-glucosidases) involved in the catabolism of dhurrin with a stain tissue system using Fast BB salt method. Additionally a LC-MS analysis will be included for analyze the dhurrin content during the development, for to see the amount and the pattern in green Sorghum seedlings and seeds, and also the amount of dhurrin in both adaxial and abaxial epidermis leaves.
Besides will be also analyzed the compounds exudate from the root hairs (dhurrin and sorgoleone) in a nutrient solution with setting up of a hydroponic system.

MATERIALS AND METHODS

Diurnal variation of dhurrin

Seeds of *Sorghum bicolor*, Sorghum sudangrass hybrid forage grasses: *S. bicolor x S. bicolor* var. Sudanense, were soaked in aerated distillated water for about 24 h at 28° C and subsequently sowed on water-saturated vermiculate in plastic boxes.

A first group were germinated in a growth chamber with 16 h photoperiod. Each day were harvested 20 seedlings. The sprouts of the seedlings were taken for analysis. The leaves was cut in smaller pieces and then the samples were put in 100 ml blue-cap bottles containing at least 30 ml of 80% MeOH. A volume of 30-50 ml 80% MeOH was added to each bottle depending on the size of the sample and boiled for 3 minutes. The boiled samples was cooled on ice. 20 µl of sample added with 80 µl of water were applied to a 0.2 µl Anapore filter to exclude major plant compounds. The samples were cleaned by centrifugation for 2 minutes at 10,000 g and 4° C. 60 µl of the filtrate was sent to LC-MS analysis. The residual filtrate was stored at -20° C.

A second group was shared in three parts, germinated in dark at 28° C for 6, 4 and 3 days respectively. Half of the plantlets were collected, the second half exposed alternatively to full light (2 days) and full dark (2 days). Each subgroup were analysed as previously described.

Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Germany) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). A Zorbax SB-C18 column (Agilent; 1.8 µM, 2.1 × 50mm) was used at a flow rate of 0.2 mL min^{-1}, and the oven temperature was maintained at 35º. The mobile phases were: A, water with 0.1% (v/v) HCOOH and 50µM NaCl; B, acetonitrile with 0.1% (v/v) HCOOH. The gradient program was: 0 to 0.5 min, isocratic 2% B; 0.5 to 7.5 min, linear gradient 2% to 40% B; 7.5 to 8.5 min, linear gradient 40% to 90% B; 8.5 to 11.5 isocratic 90% B; 11.60 to 17 min, isocratic 2% B. The flow rate was increased to 0.3 mL min^{-1} in the interval 11.2 to 13.5 min. The mass spectrometer was run in positive electrospray mode. The formic acid (HCOOH) and sodium chloride (NaCl) were used as mobile phases due to nitrogen's ability to be protonated and

glucose's ability to bind Na+ ions. It was thereby expected to see the dhurrin in the LC-MS spectra when run in positive mode due to their positive charge caused by H+ and Na+. The LC-MS results were analysed in DataAnalysis version 3.4 by Bruker Daltonics by using the function 'Extraction Ion Chromatogram'.

In situ RT-PCR of tissue sections

The in situ PCR has been done to find the specific cells which expresses CYP79A1 in young seedlings using FITC. CYP79A1 is encoding one of the enzymes in the biosynthetic pathway of the cyanogenic glucoside dhurrin in *Sorghum bicolor*.

For this experiment, the middle part of the leaves was chosen. The tissue was fixed in FAA (63% ethanol, 5% acetic acid and 2% formalin) at 4°C in 4 hours. Afterwards the plant material was washed 2 x 10 minutes with washing buffer (63% ethanol and 5% acetic acid) and once in 1 x PBS (10mM sodium phosphate and 130mM sodium chloride, pH 7,5). The tissue was then embedded in 5% low-melting point agarose in 1 x PBS and cut 85 µm thick tissue sections on a Leica VT1000S vibratome. Five tissue sections for each sample were put in 0.2 ml PCR tubes with 30 µl of sterile water containing RNase inhibitor (40U/µl) (2µl/100µl) and DNase (10U/µl)(20µl/100µl) and were left over-night at 37° C. For each tissue section a control was made to ensure that the colouring of the sample was significant and not merely background. The next day samples were washed in sterile water two time, 10 minutes. The samples were treated with 30µl of pepsin (2mg in 0,01M HCl) in 12 minutes to remove the cross linking of proteins made by FAA. The removal eases access of primers and polymerase for the future PCRs. The samples were washed two time in sterile water.

RT-PCR: for genereting cDNA from the mRNA encoding CYP79A1 the SensiScript® RT-PCR kit from Qiagen was used. To each sample 2 µl RT-buffer, 2 µl dNTP (5mM of each), 12µl H_2O and 2µl CYP79A1 revers primer (5'-ATATGAAGTTGGCGTCCTGCT-3') was added. The RT-PCR was run on JMBS 0,2 S Thermo Hybaid. The thermo cycling program was planned to 5 minutes at 65° C, 10 minutes at 4° C, 60 minutes at 37° C and finally 5 minutes at 99° C. The

first step secured that all enzymes were inactivated. During the second step 0.25 µl RNase inhibitor, 1 µl Sensiscript Reverse Transcriptase and 0.75 µl RT-buffer was added.

PCR: to incorporate digoxigenin conjugated uridine (Digoxigenin-11-uridine-5'-triphosphate; DIG-labelled dUTP) in the cDNA a PCR was made. The PCR also amplifies the copies of cDNA and thereby amplifying the signal when performing the colour-reaction. This was done by adding 2.5 µl buffer, 2 µl dNTP (5mM of each), 0.25 µl dUTP, 0.25 µl Taq polymerase (5U/µl), 20 µl H_2O and 1.25 µl of each CYP79A1 specific primers. The sequence of the foward primer and the reverse primer uses are (5'-CAAACTGCTGCTATTCGTAGTGAC-3') and (5'ATATGAAGTTGGCGTCCTGCT-3') respectively. The thermo cycling program used was 2' at 70° C, 30 x (30'' at 92°C, 30 sec. 60°C, 1' at 72°C), 5'' at 72°C.

Colour-reaction and detection: the reaction was processed at room temperature. The samples were washed 2 x 5 minutes in 1 x PBS buffer and blocked in 30 minutes in 100µl blocking buffer (0.1% acetyl, BSA in 1xPBS). For the antibody reaction an alkaline phosphatase conjugated antidigoxigenin monoclonal antibody (anti-dig) was used and diluted 1:100 in blocking buffer. The samples were incubated in 50 µl antibody mix for 1 hour. Afterwards the samples were washed 2 x 15 minutes in 10xWashing buffer (0.1M Tris-Cl, 0.15M NaCl, pH 9.5). For the colour reaction the samples were incubated in 1xWashing buffer containing 150 µm/ml 4-nitro-blue tetrazolium chloride (NBT) and 370 µg/ml 5-bromo-4chloro-3-indolyl-phosphate, 4-tuluidine salt (BCIP, 4-tuluidine salt) in about 7 minutes. The reaction was stopped with milliQ when the samples were darken than the controls and washed twice. The samples were arranged on slide and the cover slip was sealed on with transparent nail polish, then stored at 4°C. The pictures were taken using a Leica DC 300F camera and Leica DMR microscope.

Enzyme involved in the dhurrin catabolism

In plants, β-glucosidases serve a number of diverse and important function, including bioactivation of defense compounds (Nisius, 1998; Poulton, 1990; Morant *et al*., 2003; Halkier and Gershenzon, 2006; Suzuki *et al*., 2006), cell wall degradation in endosperm during germination (Leah *et al*., 1995), activation of phytohormones (Kristoffersen *et al*., 2000; Lee *et al*., 2006), and lignification (Dharmawardhana *et al*., 1995; Escamilla-Trevino *et al*., 2006).

Preliminary studies indicate that two β-glucosidases (dhurrinases) are present in different parts of the seedlings. Dhurrinase 1 appears to be in coleoptile and the hypocotyl, whereas dhurrinase 2 is found in the leaves (Hösel *et al*., 1987).

With the aim to determine the tissue localization of β-glucosidases activity two experiments has been done. In the first, in situ PCR has been used to find the specific cells where the transcript of the betaglucosidase is expressed (the methodology is described in detail in the previous experiment). In the second one, tissue sections were stained with the chromogenic substrate 6-bromo-2-naphtyl β-D-glucopyranoside (BNG) in the presence of 4-benzoylamino-2,5-diethoxybenzene-diazodium chloride hemi [zinc chloride] salt (Fast Blue BB salt). For both experiments a control was made to ensure that the colouring of the sample was significant and not merely background, in the second this was consisting in the use of Fast Blue BB salt in the absence of BNG, infact upon hydrolysis of BNG, the aglucone adheres to proteins (Cohen *et al*., 1952) and forms an insoluble complex with the Fast Blue BB salt, resulting in red/brown staining at the site of hydrolysis. BNG is an artificial β-glucosidase substrate cleaved by some β-glucosidases and has previously been used to monitor the localization of α-hydroxynitrile glucoside β-glucosidase activity in Lotus corniculatus (Rissler and Millar, 1997) and almond (Sànchez-Pérez *et al*., 2008).

The enzymes involved in the metabolism of dhurrin have been investigated in leaf blades of 7-days and 4-weeks light-grown *Sorghum bicolor* seedlings.

When the tissue is disrupted or crushed, an endogenus β-glucosidases (dhurrinase 2) catalyze the degradation of dhurrin to produce p-hydroxymandelonitrile and

then this intermediate is dissociates to give a large amount of free HCN, p-hydroxybenzaldehyde and glucose.

For this experiment the middle part of the leaves was chosen. The tissue was cut in 90 μm thick sections on a Leica VT1000S vibratome and stained with Fast Blue BB salt (20ml phoshat citrat buffer 50mM, pH 5.8 and 15mg Fast BB salt) and BNG (20mg 6-bromo-2-naphtyl β-D-glucopyranoside dissolved in 200μl of dimethylformamid (DMF).

Only Fast Blue BB salt was used for the control section. The tissue was soaked for 10 seconds in this solution and then washed twice with milliQ water. Afterwards the tissues were arranged on slide and the cover slip was sealed on with transparent nail polish. The pictures were taken using a Leica Fluorensce microscope.

Epidermal strip and allocation of dhurrin

A new method to analysed the presence and allocation of dhurrin in leaves was done in two plants of *Sorghum bicolor* at vegetative stadium: 12-weeks-old and 20-weeks-old. The analysis has been done two times in both adaxial and abaxial sections of the leaf epidermis.

To extract the epidermal strip, from each leaf a section of 4.5 cm^2 was taken and collocated on a microscope slide where some spray patch (Medical adhesive Hollister) was previously applied. After some minutes the leaf was peeled off with a few tape and the epidermis remained on the slide. Afterwards a cold extraction with 80% MeOH was done, put 200 μl of MeOH on the epidermis for 20 seconds and then 60 μl were sent for LC-MS analysis.

Distribution and activity of dhurrin metabolon

Sorghum bicolor plants “Sweet Jumbo High” and “Low cyanide” were cultivated in the greenhouse. 28 to 24 leaf samples per plant were collected from 21-weeks-old plants: a High Cyanide and a Low Cyanide. Each leaf was separated from the plant and divided above the intercalary meristem obtaining a leaf blade and a leaf sheath. The residual complex stem (length: 53,5 cm for HC and 50,3 cm for LC)

was divided in six parts. For this experiment the complex stem and the two immature leaves close to the stem were used.

Microsome preparations: all extraction steps involving the preparation of microsomes and the purification of enzyme were done at 4°C unless anything else is stated.

The samples were collected and homogenized with mortar and pestle in 5 volumes of extraction buffer (250mM Sucrose, 100mM Tricine pH 7.9, 50mM NaCl, 2mM EDTA, 2mM DTT) and 1:10 weight of tissue of polyvinylpolypyrrolidone (PVPP). PVPP was added to help the homogenization of the plant tissue and to bind the phenolics. The homogenate was filtered through a 22 µm nylon cloth and centrifuged for 15 minutes at 10,000 rpm in a Sorvall® Instruments RC5C with a FIBERLite® rotor from Priamoon Technologies Inc. This was done to remove all big plant particles such as cell debris, undamaged cells and nuclei from the supernatant. The supernatant was carefully collected and centrifuged for 60 minutes at 50,000 rpm in a L-60 Ultracentrifuge from Beckman with a 60Ti rotor. The pellet of microsomes were resuspended in 200 µl of buffer (250mM Sucrose, 100mM Tricine pH 7.9, 50mM NaCl, 2mM EDTA, 2mM DTT) and stored at -80°C.

Enzymatic assay: The samples are incubated in 30 minutes at 28°C shaking as mixture of 5 µl microsomes, 2 µl ^{14}C-Tyrosine (50µCi/ml, Amersham Biosciences), 5µl 10mM NADPH and 8µl 50mM Tricine pH 7.9. To ensure a linear reaction (the enzyme was saturated with substrate and dilution of the isotope) another reaction was carried out with the addition of 2µl 1mM L-Tyrosine and 6µl 50mM Tricine pH 7.9. After incubation 10 µl reaction was applied Silica Gel 60 F254 TLC plates (Merck) and the samples were air-dried. To focus the banda the TLC-plate was left in 100% MeOH until the mobile phase has run 1cm above the application point of the samples. After the TLC-plate was air dried before leaving in the mobile phase, which consisted of (5:1 v/v) toluene: ethyl acetate. The TLC-plate has been left air dried overnight.

Detection: The TLC-plates were processed in a phosphor-imager screen for 5 days. The radioactive-labelled compounds was depicted on the phosphor-imager screens

and visualized on a Storm 860 from Molecular Dynamics using the software Storm Scanner Control version 5.03 by Amsterdam Biosciences © 2002.

Dhurrin in seeds

This experiment has been done for to compare the dhurrin content and the HCN product on seeds and husk leaves in three varieties of *Sorghum* during different grown stages: half anthesis, milky stage and mature stage.

The three varieties used were:

- ✓ Ark3048, low tannin content, eaten by birds
- ✓ P72, low tannin content, do not eaten by birds.
- ✓ Sudan IV, high tannin content, do not eaten by birds.

Each samples consisted of five seeds and the extraction was made with MeOH (80%), boiling for 3 minutes.

It has been done the comparison of betaglucosidase activity in different parts of seed, both to take in account the endogenous quantity and, as an alternative, adding 30 nanomoles of dhurrin. For the experiment the seeds were divided in parts: embryo, endosperm, pericarp and hull. The extraction was made with MeOH (80%), boiling for 3 minutes.

For both experiments the boiled samples were cooled on ice. Then 20 µl of sample were added with 80 µl of water and filtred with a 0.2 µl Anapore filter by centrifugation for 2 minutes at 10,000 g, 4° C. 60 µl of the filtrate were sent to LC-MS analysis.

Determination of compounds exudated from the root: sorgoleone and dhurrin

Seeds of *Sorghum bicolor* were soaked in distillate water for about 24 h at 28°C and subsequently sowed on water-saturated vermiculate in plastic boxes. After 4 days, the plantlets were transplanted on an hydroponic system, consisting in modified pots. Each pot was filled with 1L of modified Hoagland's nutrient solution. The oxygenation of the solution was guaranteed by a pump that injected air full time. Once a week the solution was replaced with new one and 30 ml were

taken for analysis. A quote of 5-10ml of ethyl acetate was added to the 30 ml of old solution, the mix was shacked for few minutes, then allowed to stand for some minutes. After stabilization two phases were obtained, and the upper phase was taken for centrifugation. This was performed in a freezedry Centrifuge for vacuum Concentrator (Scan-speed 32 teflon). Once the solution was completely evaporated, the powder was suspended in 200 µl of MeOH and 60 µl were sent for LC-MS analysis. The plants were cultured in hydroponic for 8 weeks and the experiment repeated twice.

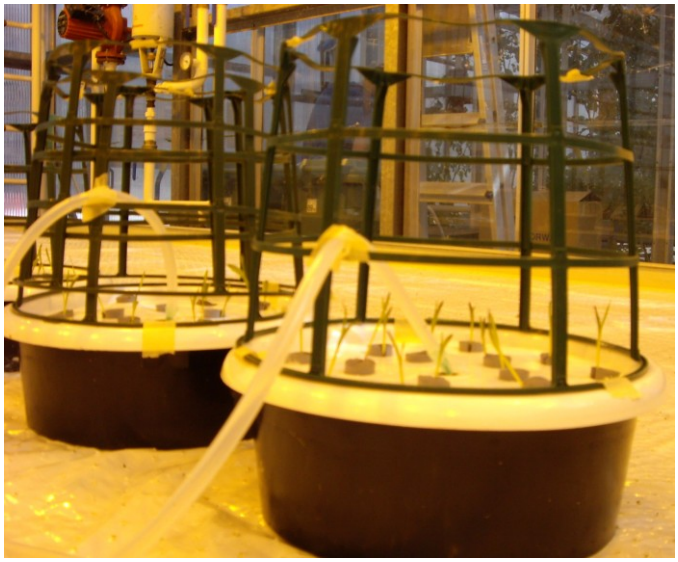


Fig. 12 Hydroponic system

Fig. 13 Sorghum seedlings 2 and 6 weeks-old

At the end of cultivation, roots and aerial part were analysed to check the amount of dhurrin in tissues. The leaves and the roots were cut in smaller pieces and the samples were put in 250 ml blue-cap bottles containing 50 ml of 80 % MeOH. A volume of 50-200 ml 80% MeOH was added to each bottle, depending on the size

of the sample, then boiled for 3 minutes. The boiled samples were cooled on ice and 20 µl of sample added with 80 µl of deionized water were applied to a 0.2 µl Anapore filter to exclude major plant compounds. The samples were cleaned by centrifugation for 2 minutes at 10,000 g and 4° C. 60 µl of the filtrate was sent to LC-MS analysis. The residual filtrate was stored at -20° C.

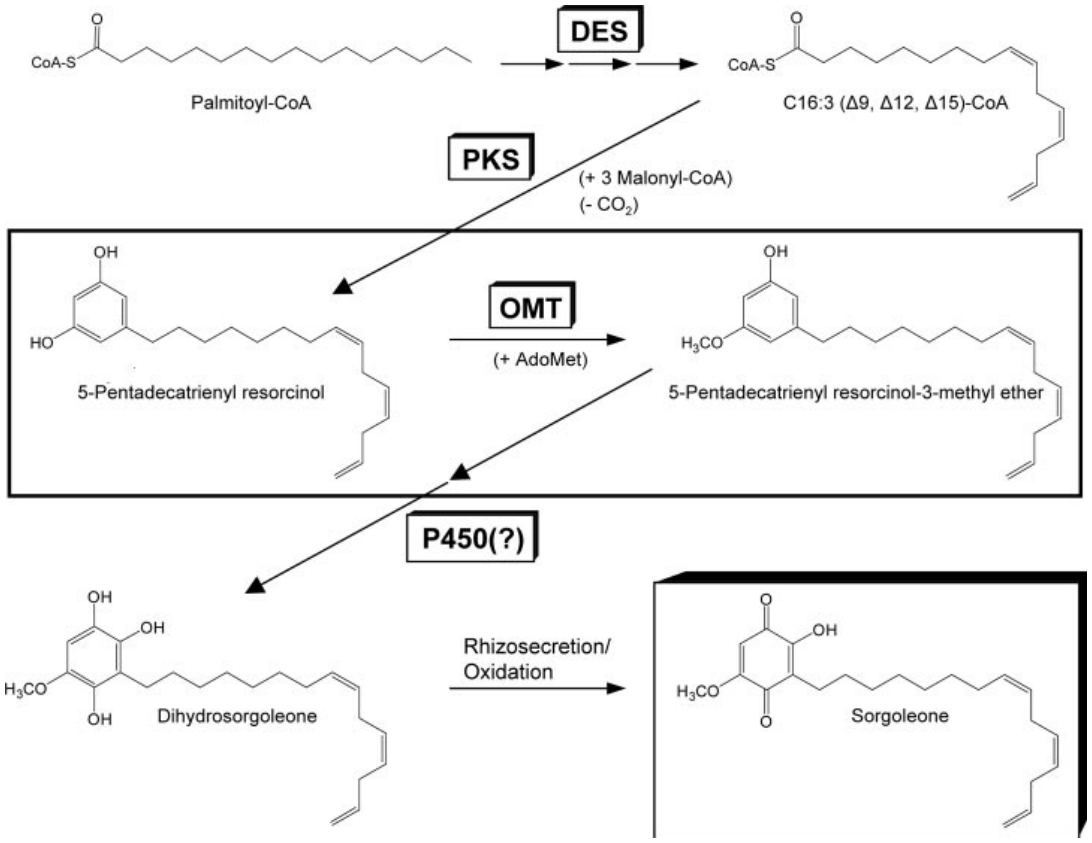


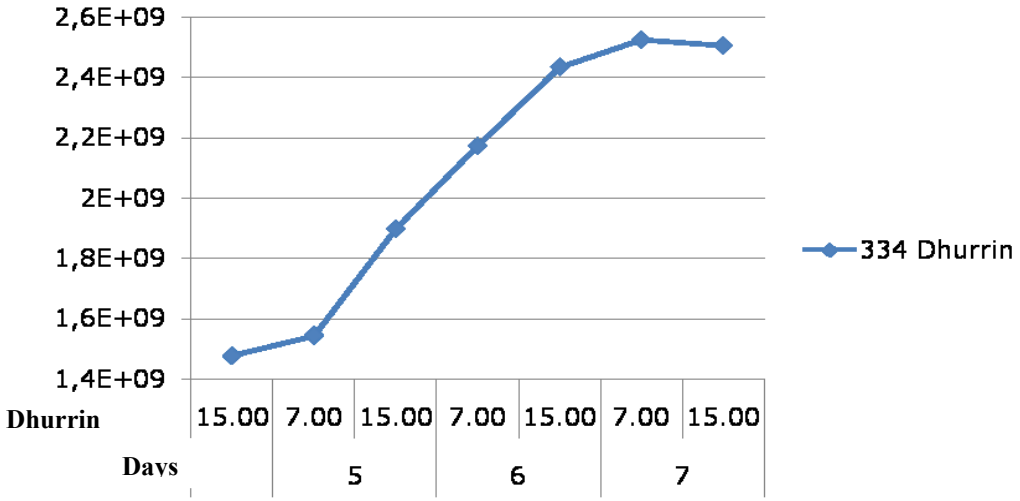
Fig. 14 Sorgoleone pathway (Baerson *et al*., 2008)

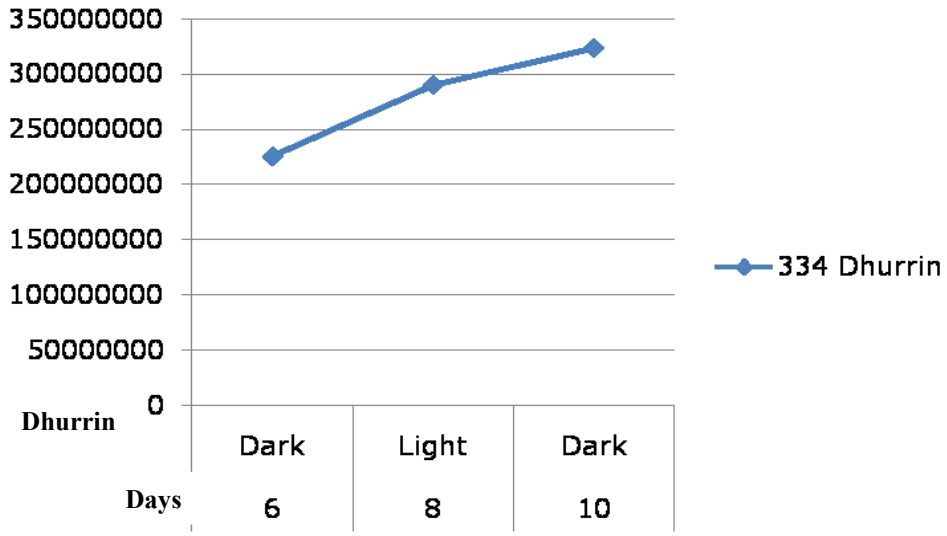
RESULTS AND DISCUSSION

Diurnal variation of dhurrin

The production of dhurrin tends to increase exponentially during germination and no differences were observed among treatments or among repetitions. The synthesis-accumulation occurred equally in light and dark periods. Even for seed treated in dark all day long. The trend has been similar in an experiment performed by Steve & Adewusi (1990), although the experimentation shows that the rate in dark was faster.

The same pattern was also seen when the seedlings were left to a period of full dark and full light.



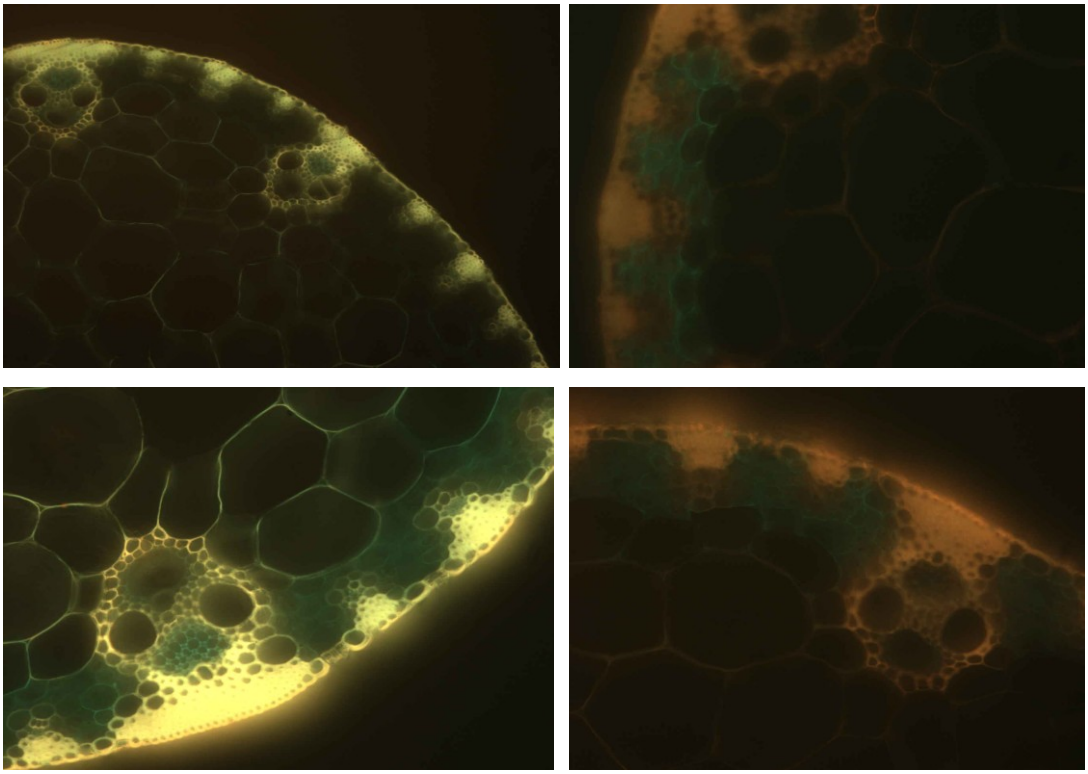


In situ RT-PCR of tissue sections

With this experiment on tissue sections it has been possible to locate the mRNA encoding CYP79A1. The reaction was concentrated around the epidermis, the cell wall and the bundle sheath parenchyma cells. No colouring have been obtained in the non treated cells. The control shows the background, and that was of importance to clearly identify the tissues and false positive staining.

Control

CYP79A1

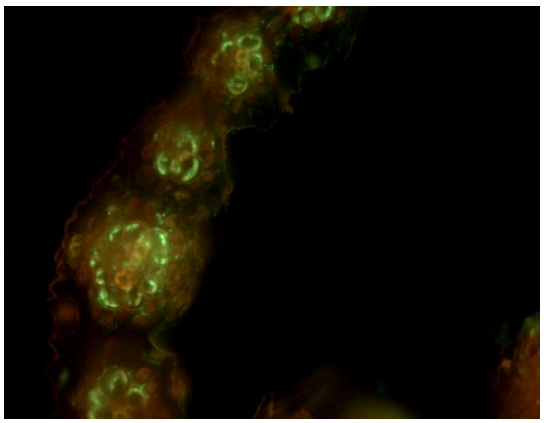


UGT85B1

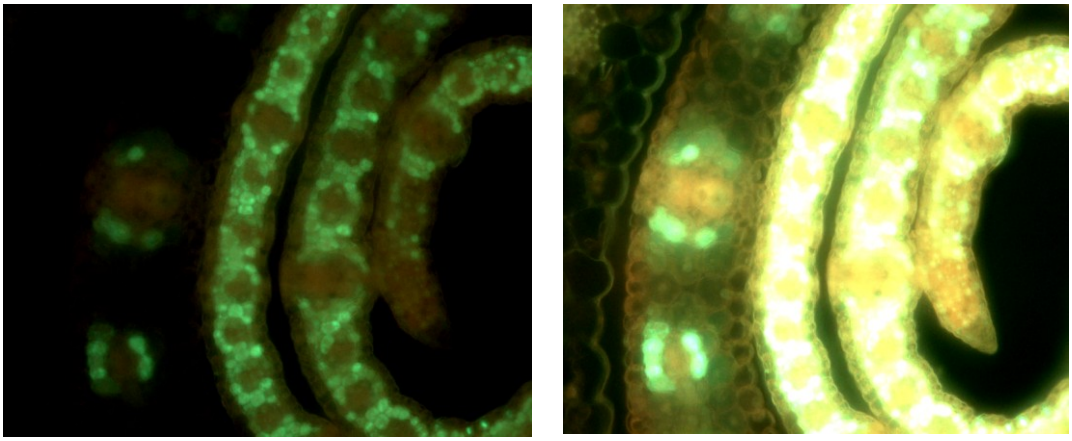
CYP71E1

Enzyme involved in the dhurrin catabolism

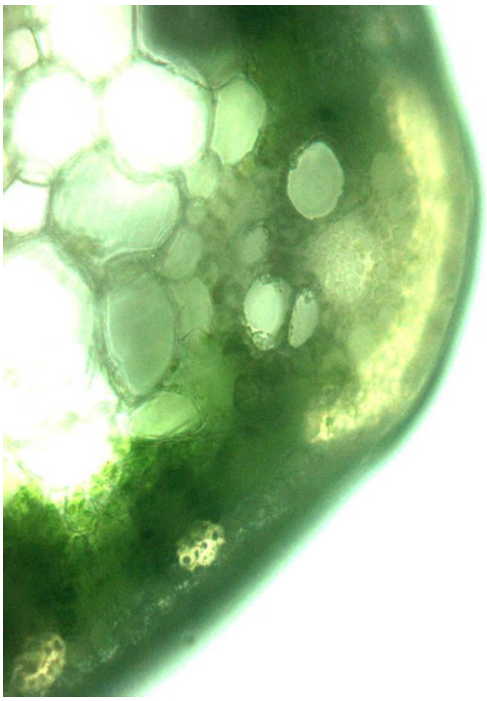
The distribution of dhurrinase was investigated in light grown leaves sections. In the in situ PCR method we identify in which cells we have the transcript. With the Fast blue BB salt method we observe in which cells the enzyme is active.The results showed that the highest activity was located in the vascular tissue and sheath bundle cells, whereas no activity was detected in epidermal cells. No colouring have been obtained in the non treated cells in the control with in situ PCR. No or very weak color development was observed upon the addition of Fast Blue BB salt in the absence of BNG, demostrating that the chromogenic reaction is dependent on the presence of the BNG aglucone formed by β-glucosidases hydrolitic activity.



background



Dhurrinase2 expression in 7 days old sorghum grown in light



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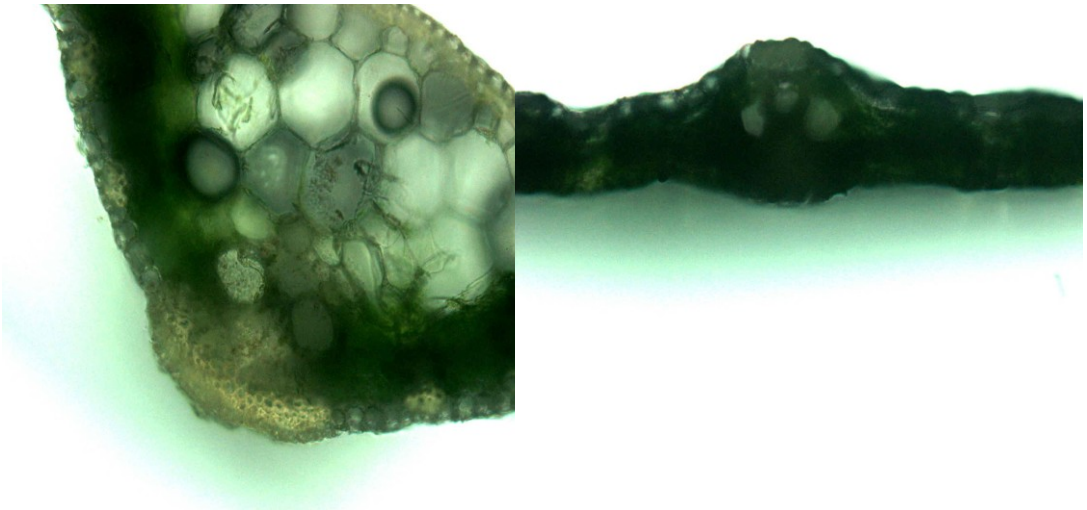
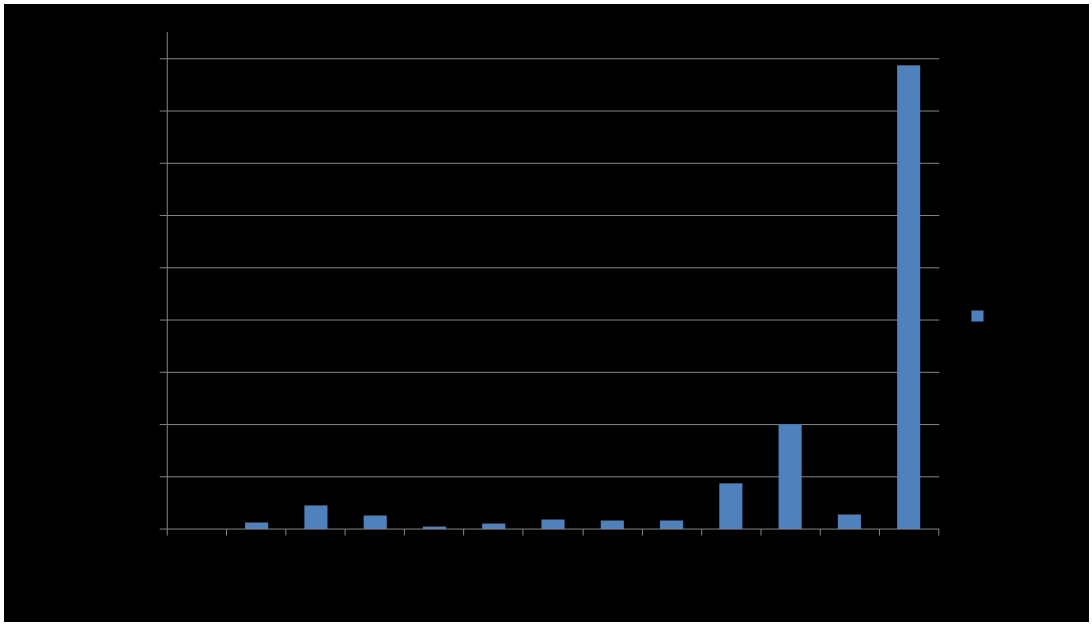


Dhurrinase2 expression in 4 weeks old sorghum grown in light

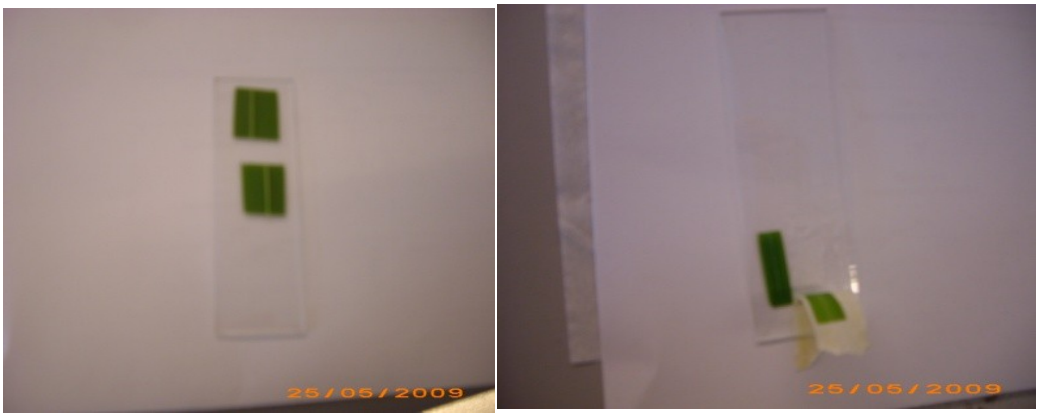
Allocation of dhurrin by epidermal strip

The distribution of dhurrin in leaves showed an increasing trend from bottom to top. The leaf number one, the bottom leaf of the plant, showed a total of dhurrin equivalent to zero, probably for the reason that it was completely desiccated. The other leaves showed an increasing trend, even if the quantity was low. The content was correlated to the age of the leaves, and to the absence of new synthesis of the compound. Only in the young innermost leaves at the top, there was a most important quantity of dhurrin.

Graph epidermal strip



Epidermal strips



Epidermis

Distribution and activity of Dhurrin metabolon

In their experiments, Halkier and Møller (1989) demonstrate that metabolic activity was located in microsomal preparations, obtained from young developing tissues. The microsomal preparation shows the activity of the dhurrin metabolon and the tissue in which the synthesis take place. Since the last enzyme in the pathway is lost during the preparation of microsomes, the end product will be p-hydroxybenzaldehyde. The degradation of the unstable intermediate p-hydroxymandelonitrile produce p-hydroxybenzaldehyde. The presence of p-hydroxybenzaldehyde thereby indicates the activity of the metabolon.

No activity has been detected in the microsome preparations. Looking the TLC plates it have been evident that the reaction stops to the Tyrosine. The biosynthesis seems take place only in the stage among young developing tissue and mature tissue. Then the synthesis stops and dhurrin is stored into the cells.

Fig. 15 Sorghum bicolor low cyanide content

Fig. 16 Leaves and complex stem

Fig. 17 Sorghum bicolor high cyanide content

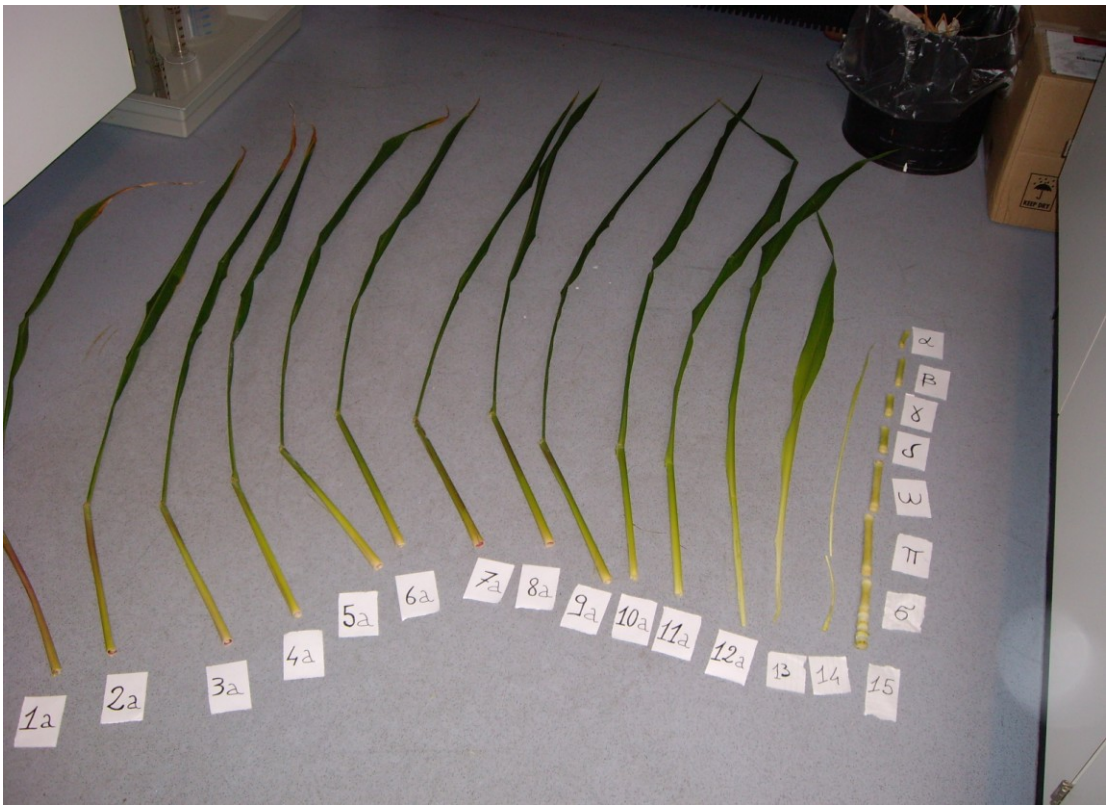
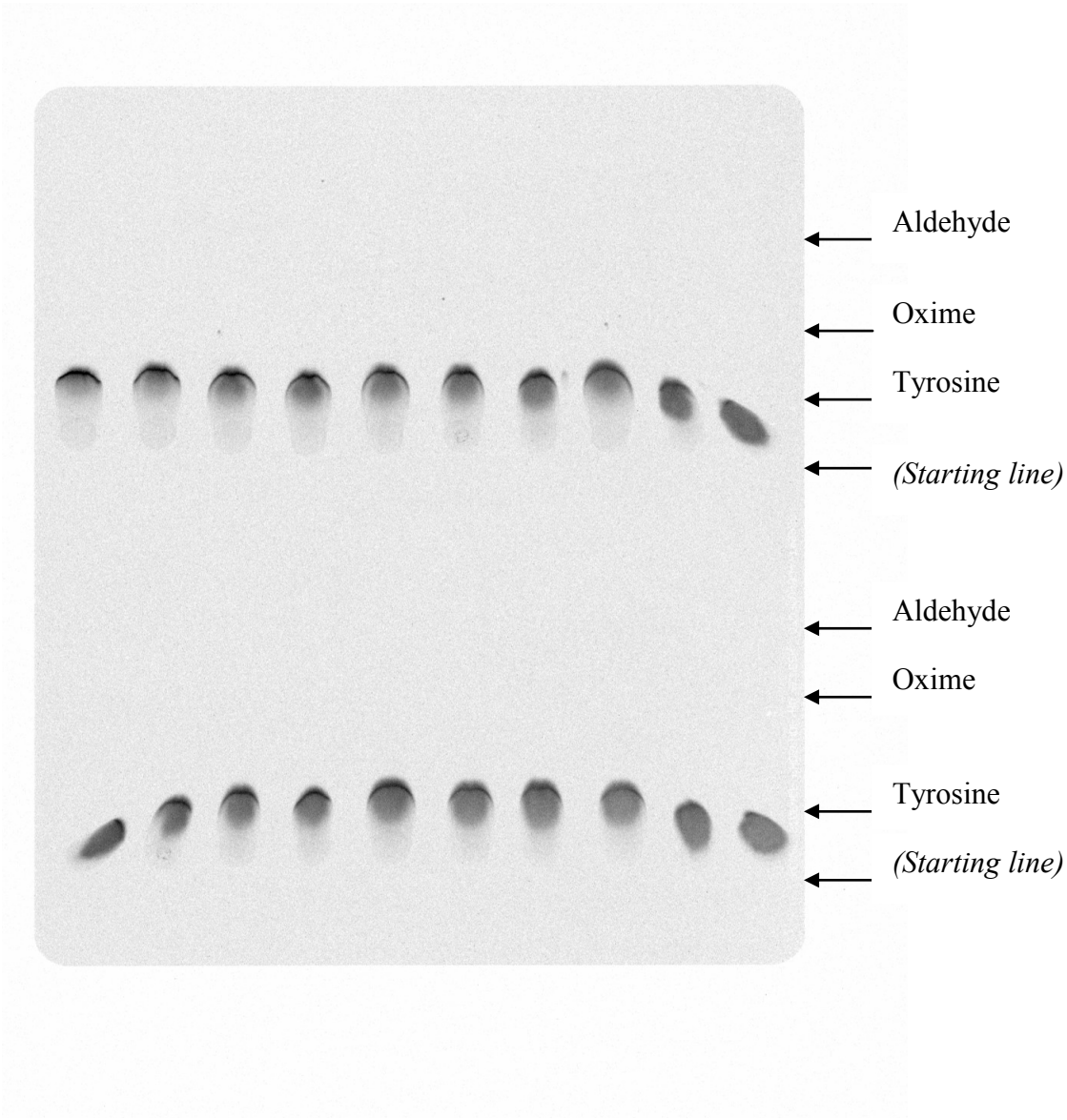
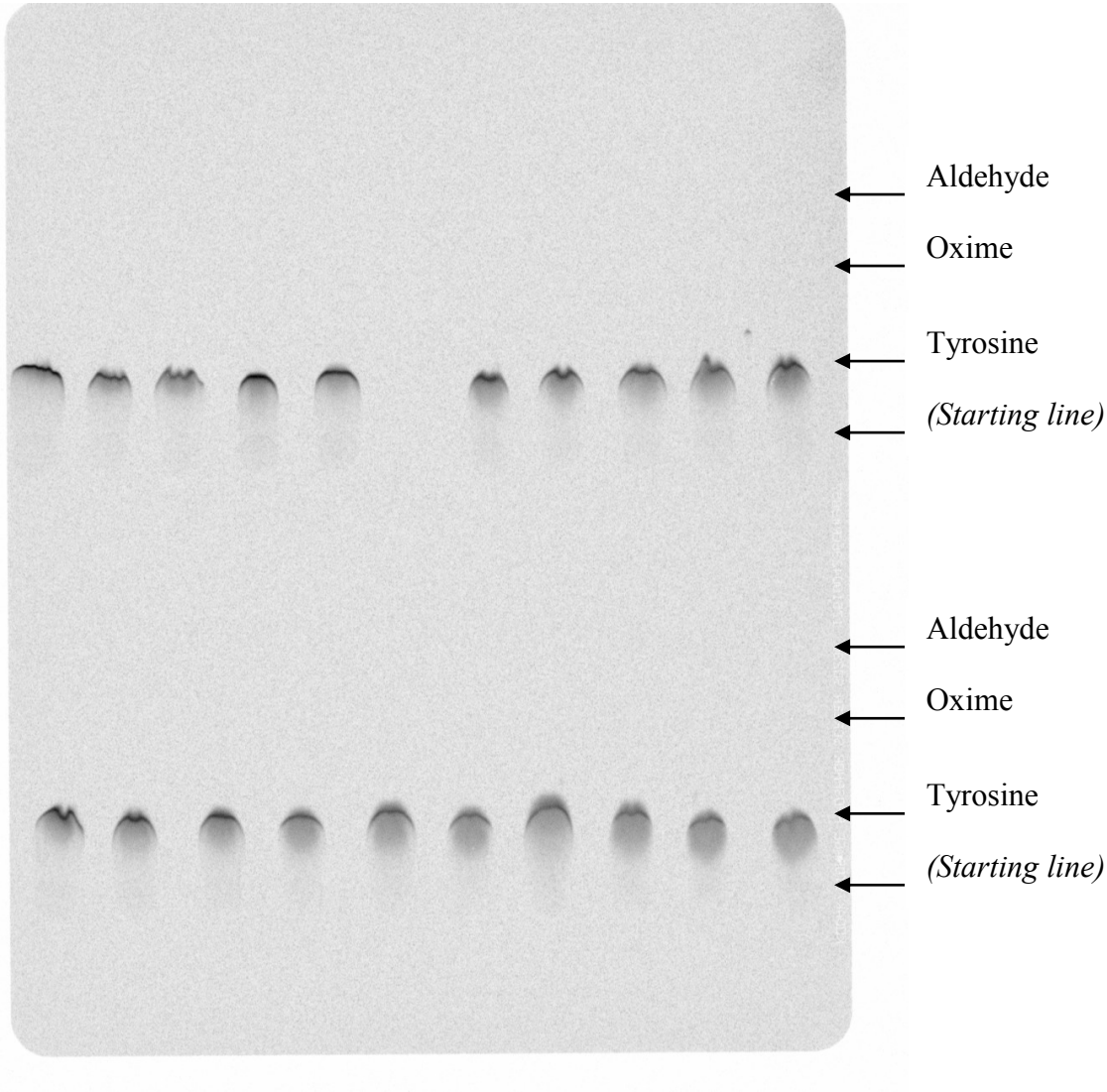
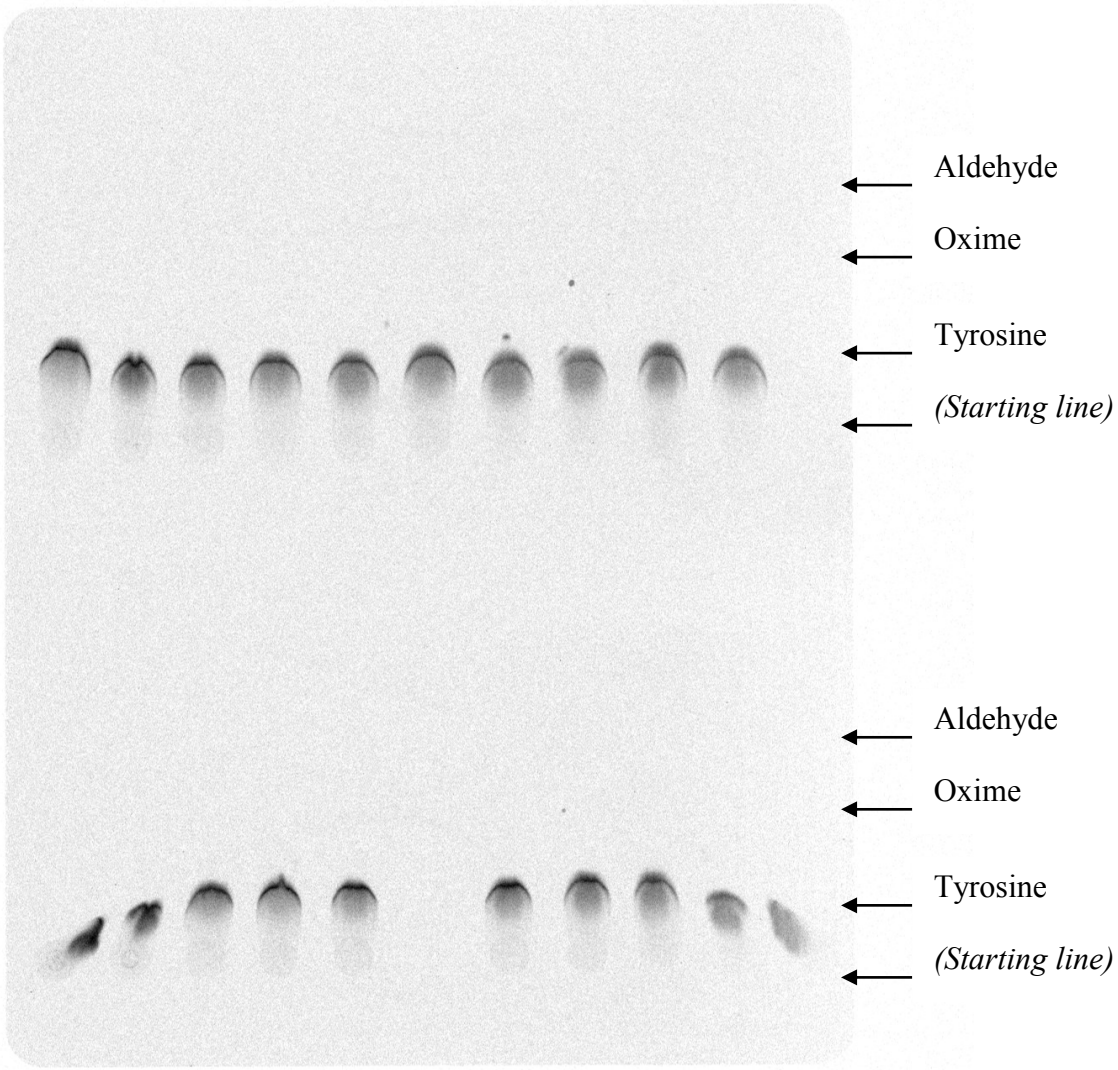


Fig. 18 Leaves and complex stem

Activity of the dhurrin-metabolon




Aldehyde
Oxime
Tyrosine
(Starting line)
Aldehyde
Oxime
Tyrosine
(Starting line)


Aldehyde
Oxime
Tyrosine
(Starting line)
Aldehyde
Oxime
Tyrosine
(Starting line)

Dhurrin in seeds

Analyzing the dhurrin content of three sorghum varieties during seed development, it was possible to see that the higher quantity of dhurrin was product during the milky stage. Among varieties, the cultivar P72 shown the higher content.

A similar trend was observed analyzing the dhurrin content in the husk. The major quantity was product during the milky stage. In that case, the higher content was detected in the cultivar ARK3048. A good production was observed during the half anthesis in the same variety. Absence of dhurrin was seen during all seed developmental stages in Sudan IV.

The endogeneous breakdown of dhurrin by beta-glucosidase was detected as content of HCN. The higher lowering was observed in the milky stage in the cultivar Sudan and P72. No reduction was seen in P72 during the half anthesis.

In the husk the breakdawn was regular in the half anthesis, an increment was seen during the milky stage and reduction during the mature seed stage. Away from each other a sample of Sudan produced a high quantity of HCN. Absence of production has been observed in P72 during half anthesis.

The addiction of 30 nmol of dhurrin, compared with the untreated, was evidenced by betaglucosidase activity in embrio/endosperm, hull and pericarp. In the untreated, it have been seen that the availability of substrate was higher during the mature seed stage (embrio/endosperm) and during the half anthesis-mature seed (hull). Afterwards the addiction of 30 nmol of dhurrin, no increase betaglucosidase activity was observed. As regarding the pattern, in the pericarp the availability of substrate was higher in the half anthesis stage and increased when 30 nmol of dhurrin were added. Then in the first two cases also if us added more substrate, the betaglucosidase works in the same manner, whereas in the third case increases its activity.

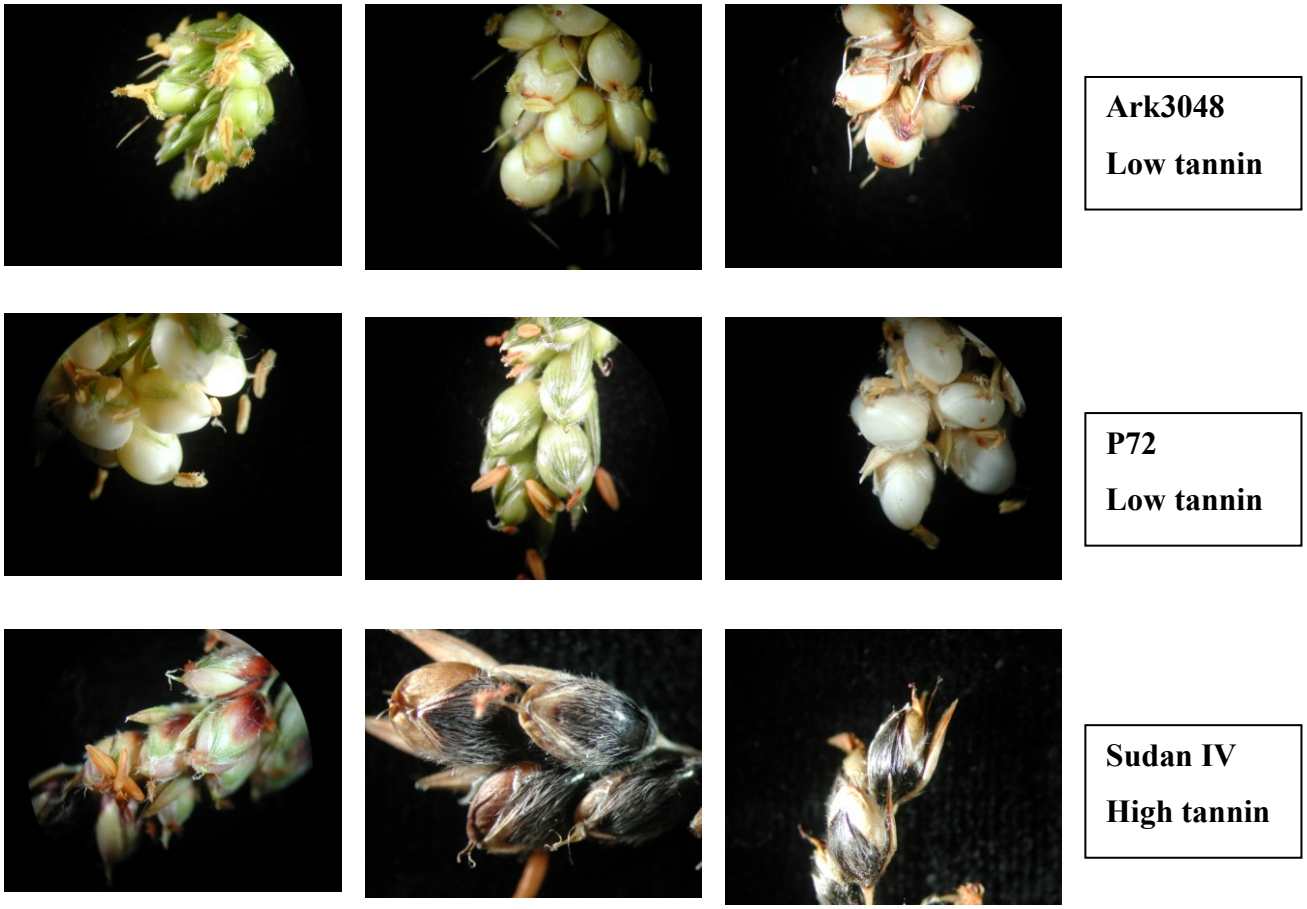
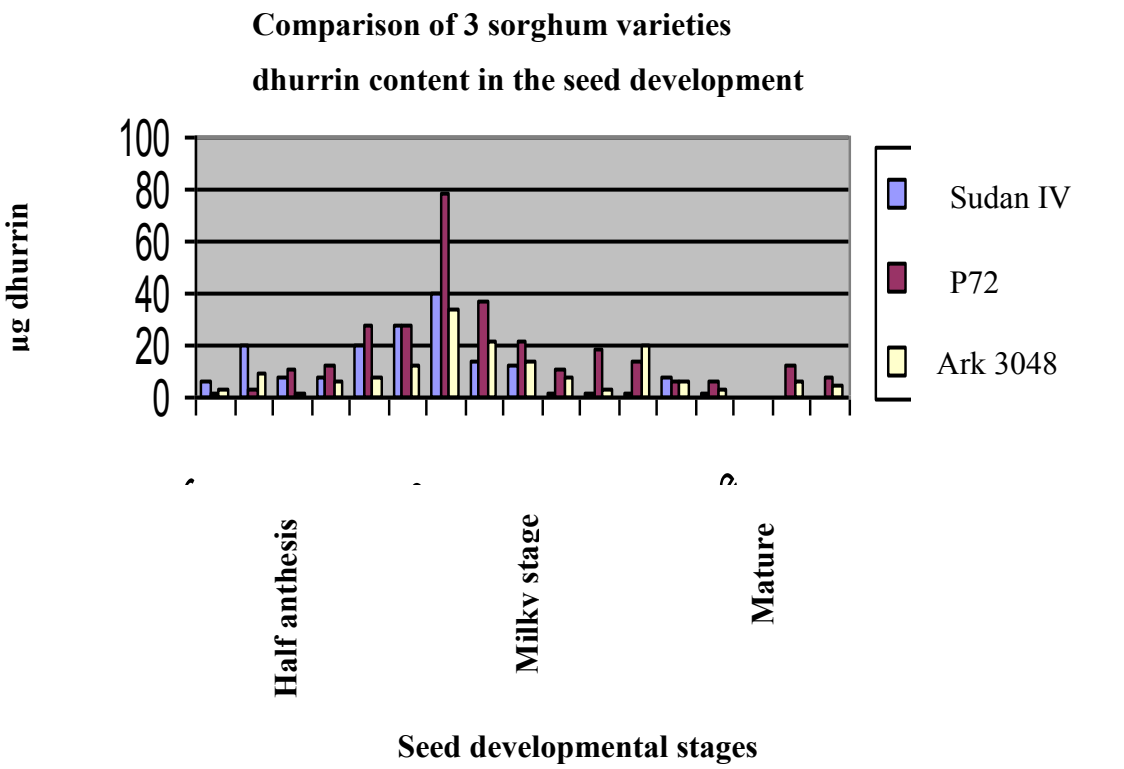
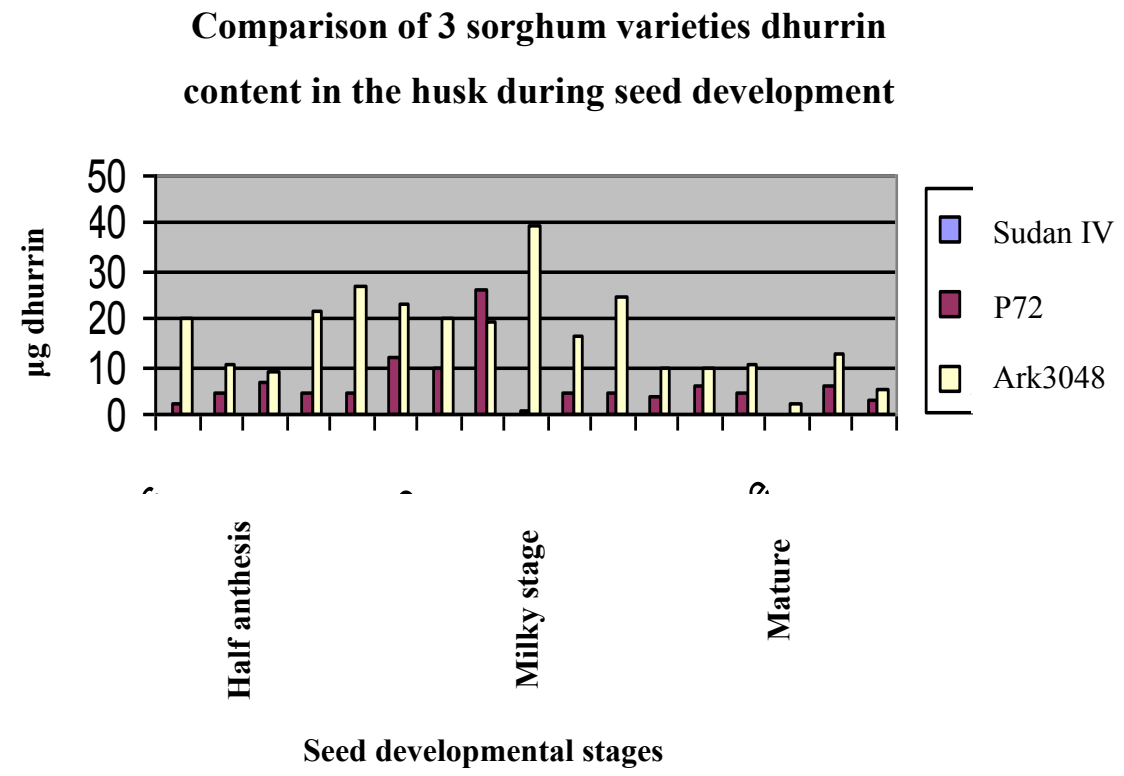


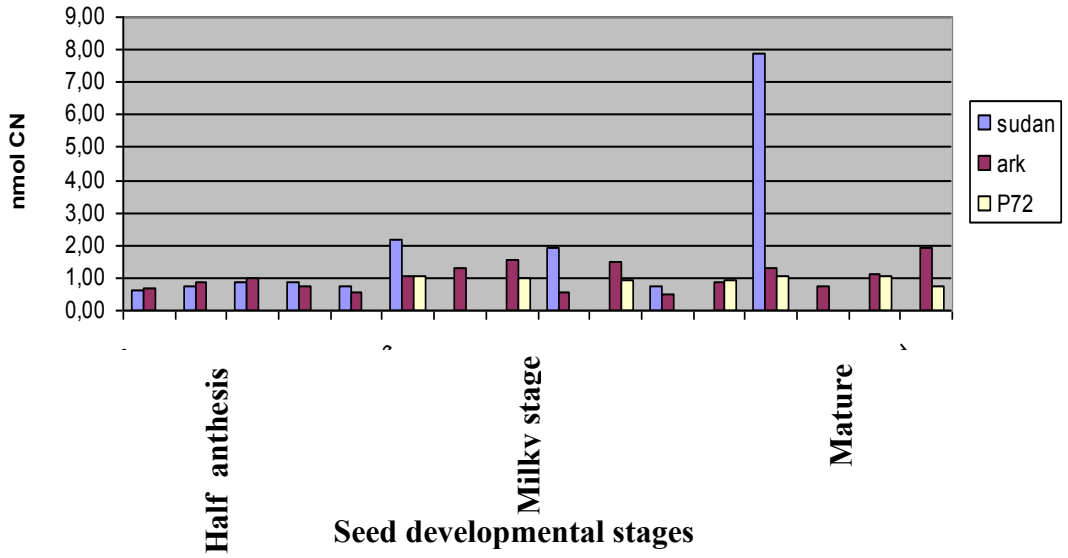
Fig. 19 Sorghum variety and developmental stages.

Giovanni G. Barmina. Metabolites produces during different growth conditions of Sorghum bicolor (L.) Moench. Tesi di dottorato in Produttività delle Piante coltivate, Università degli studi di Sassari.

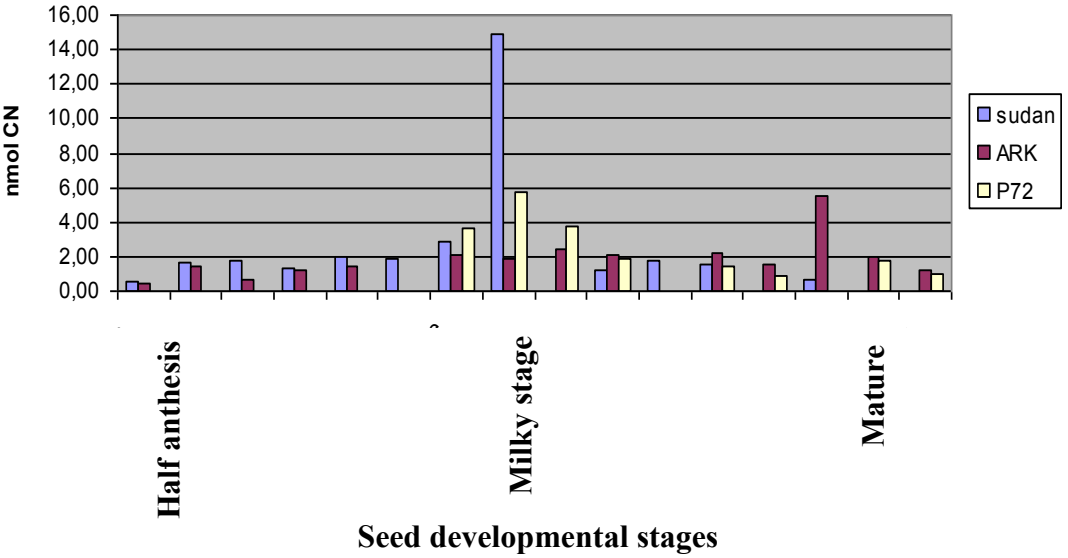

Comparison of 3 sorghum varieties
dhurrin content in the seed development
µg dhurrin
100
80
60
40
20
0
Sudan IV
P72
Ark 3048
Half anthesis
Milkv stage
Mature
Seed developmental stages


Comparison of 3 sorghum varieties dhurrin
content in the husk during seed development
µg dhurrin
50
40
30
20
10
0
Sudan IV
P72
Ark3048
Half anthesis
Milky stage
Mature
Seed developmental stages

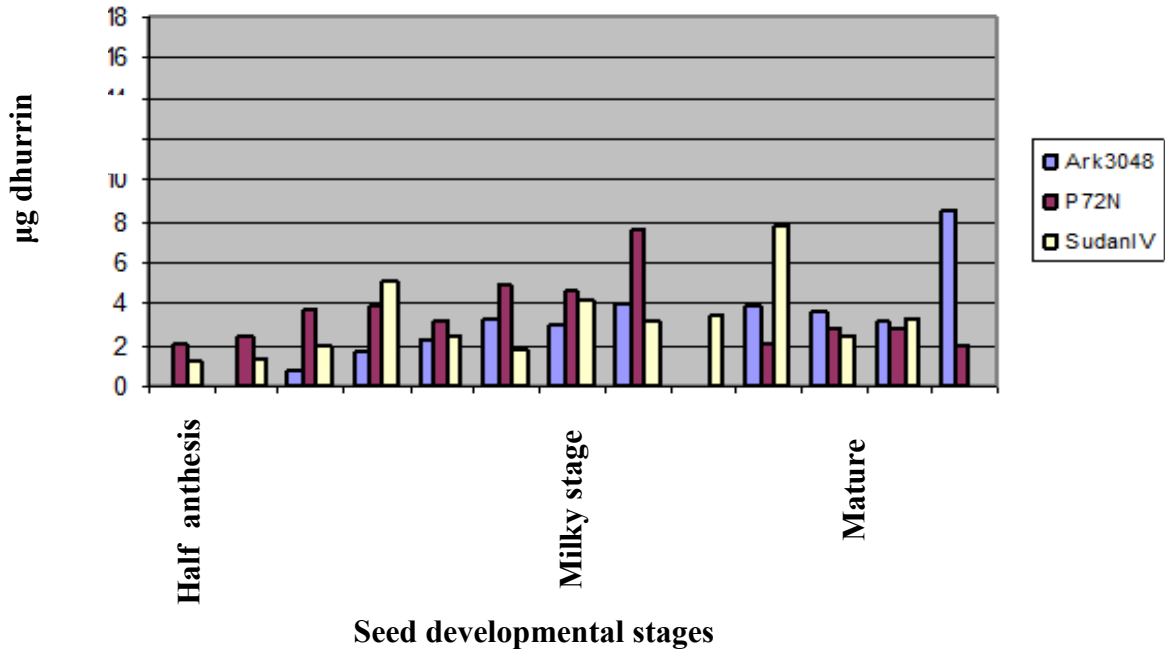
The endogenous breakdown of dhurrin by beta glucosidase in the husk during seed development



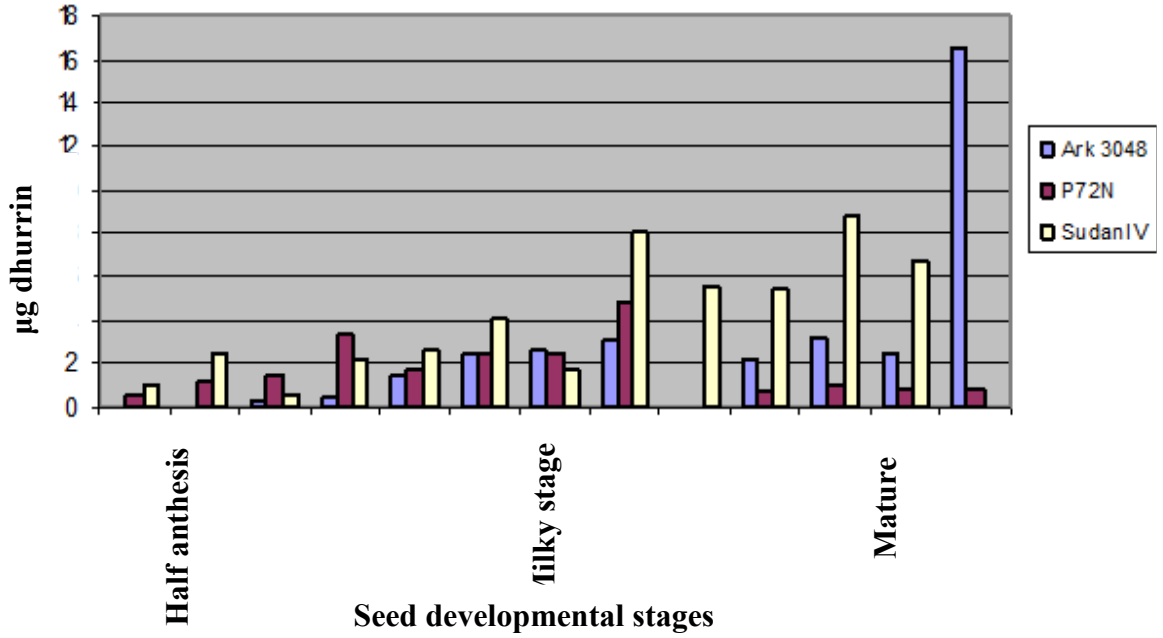
The endogenous breakdown of dhurrin by beta glucosidase in seed under development



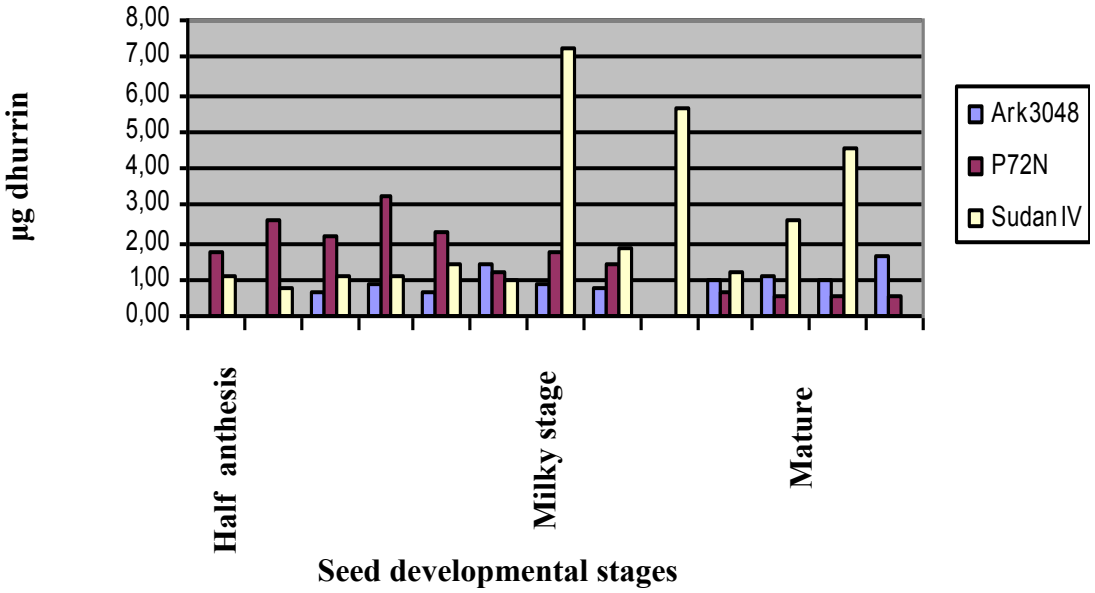
Comparison of endogenous beta glucosidase activity embryo/endosperm when added 30 nmol dhurrin



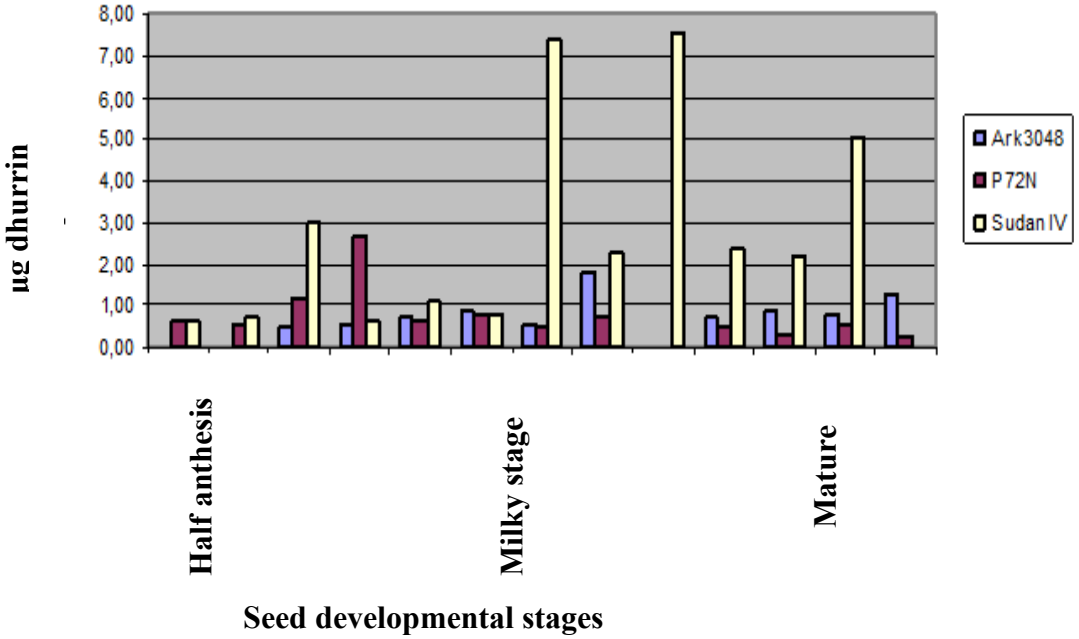
Comparison of endogenous beta glucosidase activity in the embryo/endosperm



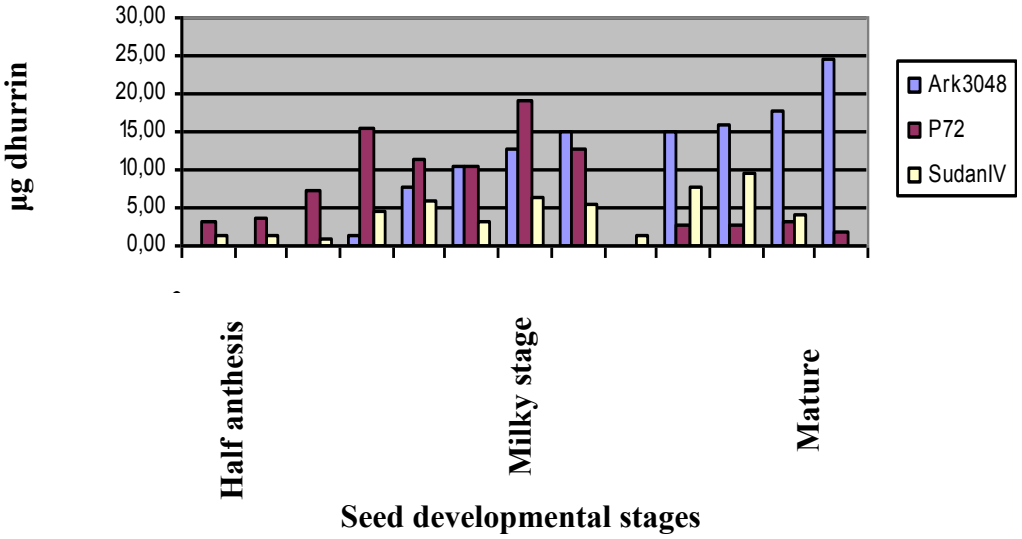
Comparison of beta glucosidase activity in the hull when added 30 nmol dhurrin



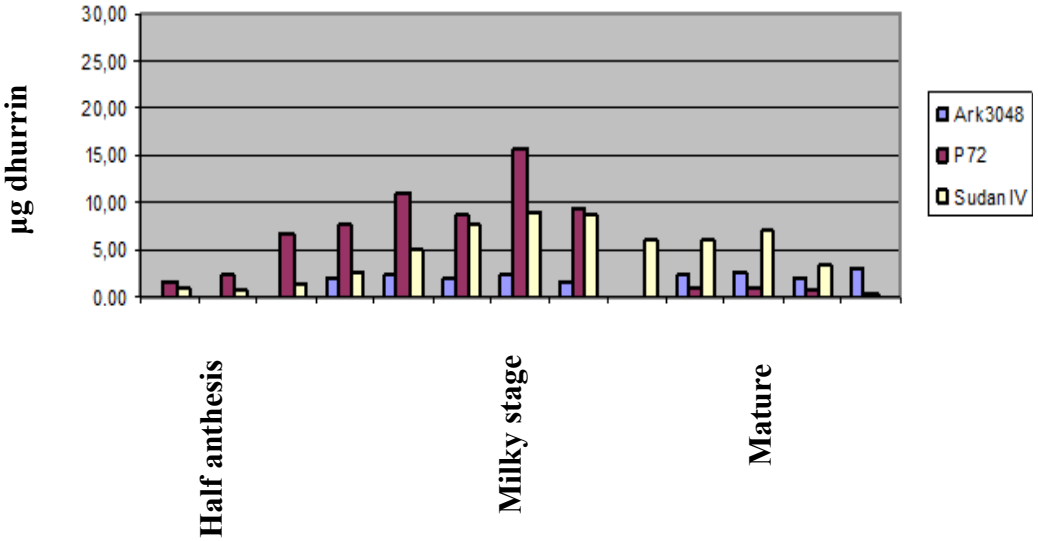
Comparison of endogenous beta glucosidase activity in the hull



Comparison of beta glucosidase activity in the pericarp when added 30 nmol dhurrin



Comparison of the endogenous beta glucosidase activity in the pericarp

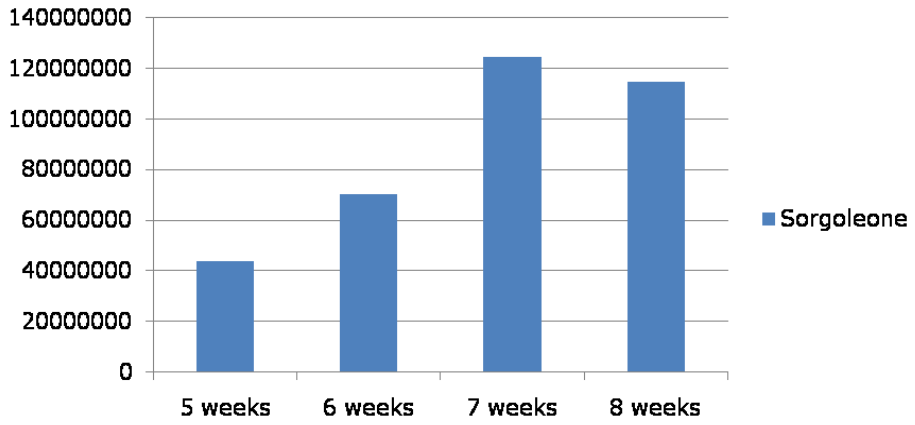


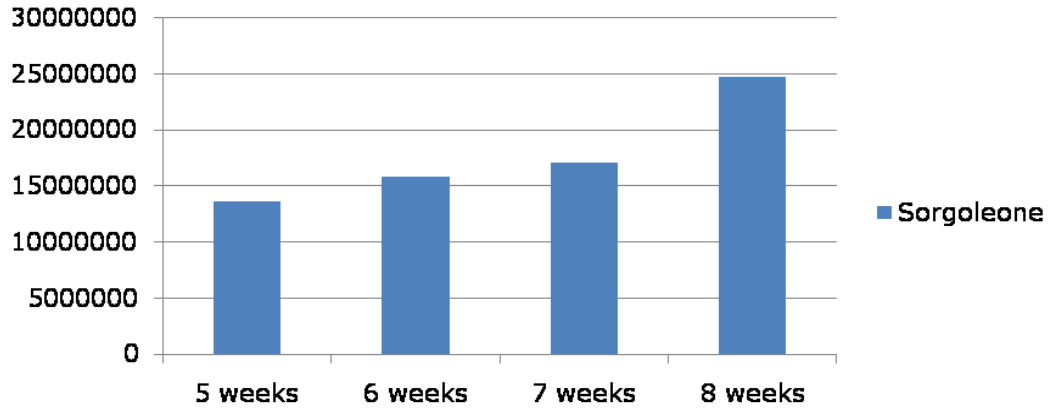
Production sorgoleone and dhurrin exudates by roots

Analyzing the nutrient solution in the pots during cultivation, it has been seen that the quantity of sorgoleone tends to increase during the growth of the plant.

The analisys have been done on young plants, under 8 weeks, because growth of the plants stops due to the limited space of pots and the big size of leaves and roots. For that we did not detected the compound when the plant grows.

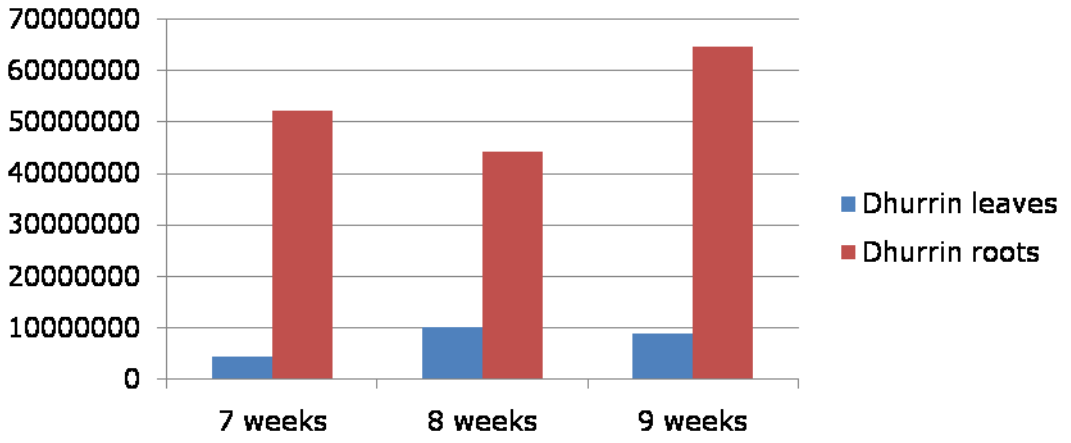
Sorgoleone

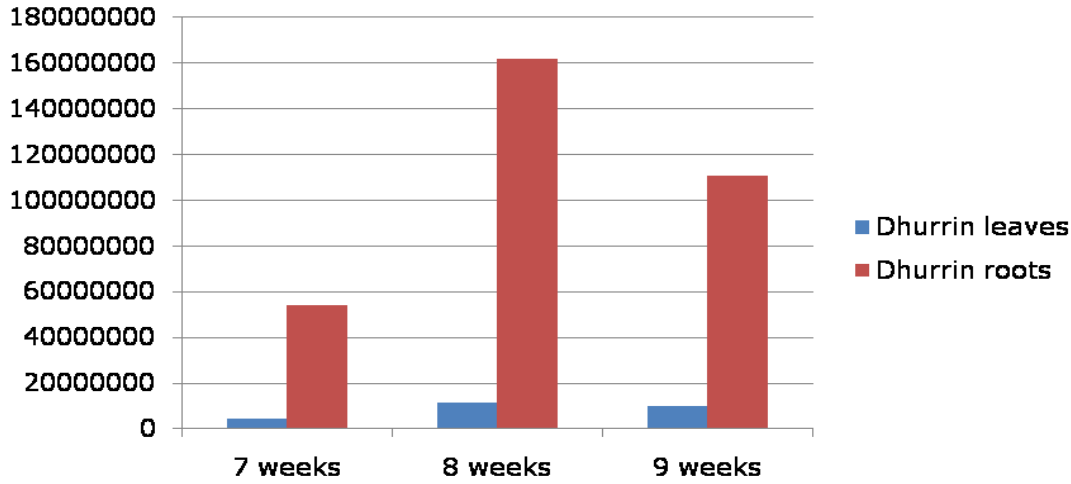




Dhurrin in roots and leaves

It has been seen that the quantity of dhurrin is higher in the roots, but the pattern is variable during this different periods. It should be of interest, to analyze if there is a translocation of dhurrin from the arial part to the roots or if there is a synthesis of this compound in the roots.





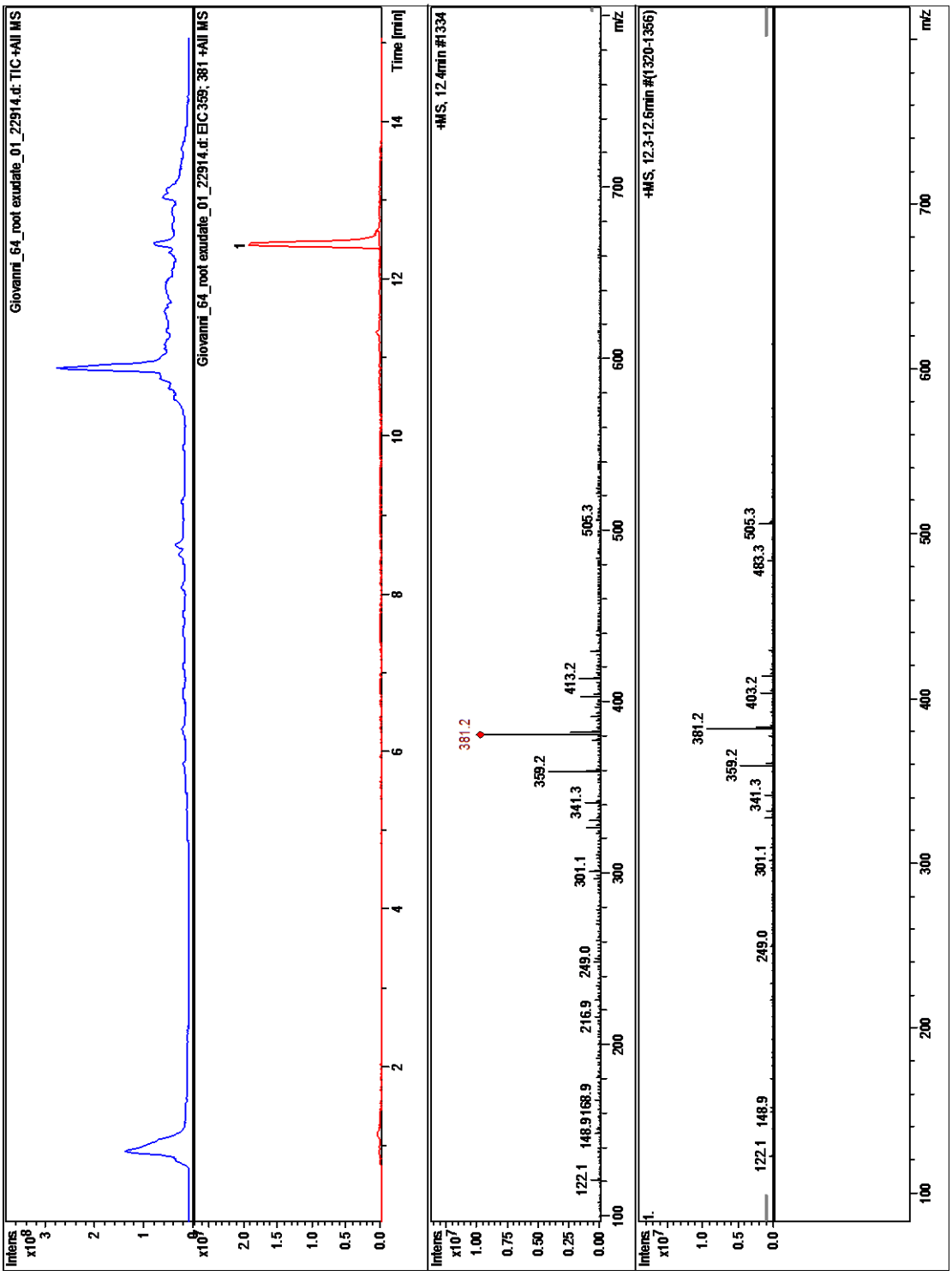


Fig. 20 Sorgoleone chromatogram

CONCLUSION

Plant secondary metabolites are responsible for many of the properties of plant and are widely employed either directly or indirectly in a large number of pharmaceutical industries. These are, also, of primary importance in food compounds (pigments, fragrances etc.), fibre production, horticultural etc. It is of increasing evidence that many of these compounds are involved in plant defence as the cyanogenic glucosides in *Sorghum spp*.

In this work it have seen that the dhurrin content in seedlings is higher during the first period and it is accumulate in the tissues. The dhurrin production has been seen also in the seeds during the different developmental stages. When the plant grow up, the production of dhurrin stops. That was confirmed analyzing the metabolic activity with microsomal preparation in old plant: no activity was seen. Even analyzing the epidermal strip, the higher content of dhurrin was found in the youngest leaves. These result confirms that the plant synthesizes dhurrin to protect itself when it is young and the tissue are weak and more easily attacked by pests. When the plant grows up the synthesis is stopped and the dhurrin rests accumulated in tissues.

The higher quantity of dhurrin is stored in the epidermis, while the enzyme is present in the vascular tissue and sheath bundle cells. So that, in plants there is not contact among enzyme and dhurrin, and the HCN is formed and released only in consequence of an attack by pests. So that, it is been determined that the production of cyanide is used in plants as defence, as mix of enzyme and dhurrin. The synthesis of dhurrin metabolon was located in the young developing tissue by labelling CYP79A1. The complex enzyme was present in the epidermis cells and bundle sheat parenchima cells.

Moreover, the sorghum plants produce sorgoleone as defence from the surrounding plants. The sorgoleone is product during the growth of the plant, probably during the whole life cycle. In the next experiments, it will be pointed out if there is a traslocation from the leaves to the root, as seen for dhurrin, or it is synthesized directly in the roots.

The study of secondary metabolites give the advantage to reveals the synthesis, accumulation and distribution in the plant. Moreover, it should be a base useful for study and production of pesticides based on plant physiology, having a better interaction with the enviroment, and suitable for use in organic agriculture.

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