PLANT REGENERATION AND HYPOXOSIDE CONTENT IN
HYPOXIS OBTUSA

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ABSTRACT.—Three *Hypoxis* species known to produce active glucosides were tested in order to assess their potential for in vitro propagation and tissue culture manipulation. Plant regeneration was achieved from the rhizome of wild type plant of *Hypoxis obtusa* on Murashige and Skoog medium supplemented with 1 mg/liter 6-benzylaminopurine (BAP). The other *Hypoxis* species tested (*Hypoxis nyasica* and *Hypoxis angustifolia*) failed to regenerate under the same culture conditions. The content and distribution of the main active principle, hypoxoside, was determined in parental and regenerated plants of *H. obtusa* by hplc.

Hypoxis species (Hypoxidaceae) are herbaceous plants with a tuberous rhizome and radical leaves longer than the inflorescence. The genus occurs mainly in the Southern hemisphere, with centers in South America and Central and Southern Africa. Parts of these plants, especially the rhizomes, have been used for many centuries in African popular medicine to treat several diseases (1). In Mozambique *Hypoxis obtusa* Bush is well known as a medicinal plant under the local name of "Chirranga buharu," and its rhizome is directly dispensed by local "curanderos" (healers) for treatment of urinary diseases (2). In Malawi the same use, as well as for treatment of prostate hypertrophy and internal cancer, has been reported for *Hypoxis nyasica* Bak.[1]

A search for the active principle led to the isolation and chemical characterization of various glucosides from the fresh rhizomes of *Hypoxis* species. These compounds contained an uncommon aglycone structure consisting of C_6(aromatic)-C_3-C_2-C_6(aromatic). The main components of the glucoside fraction

were hypoxoside (2) and nyasoside (3). Hypoxoside has also been found in other *Hypoxis* species as well as the related species *Spiloxena schlechteri* (4).

The glucosides possess low toxicity (the rhizomes are also used as food) (4), and the aglycones possess antiphlogistic (5), bacteriostatic, and bactericidal properties (6) as well as significant activity against malignant KB cells and P-388 doxorubicin resistant and sensitive lines (7). Clinical investigations have led to the registration of several patents (6–9) and the commercialization of the extract of *Hypoxis rooperi* in Germany under the trade name "Harzol" for the treatment of prostate adenoma (10).

In consideration of the medical importance of these plants and the difficulty of their propagation by seeds which appear to be dormant, an investigation was undertaken in order to test the possibility of in vitro propagation of some *Hypoxis* species. In this paper we report the results of such a study, together with the determination of hypoxoside distribution in different organs of *H. obtusa*.

RESULTS AND DISCUSSION

TISSUE CULTURES.—Among the

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three different *Hypoxis* species tested, only explants from *H. obtusa* rhizomes were successfully cultured and propagated (Table 1). No further attempts at propagation were carried out with *H. nyasica* and *Hypoxis angustifolia* H.M.

cytokinin (BAP) containing media. The same response was observed, though to a lesser degree, when explants were cultured on hormone-free Murashige and Skoog medium (MSO). In both cases, the shoots developed within 1 month of

TABLE 1.   Growth Response of Rhizome Explants from *Hypoxis obtusa*, *Hypoxis nyasica*, and *Hypoxis angustifolia* on MS Basal Medium, Supplemented with Different Hormones.[a]

Hormone	*H. obtusa*	*H. nyasica*	*H. angustifolia*
none	shooting	no response	no response
NAA[a] 5 mg/l } BAP[b] 1 mg/l }	slight callus formation	no response	no response
2,4D[c] 3 mg/l } Kin[d] 0.2 mg/l }	callus formation (cut ends)	slight callus formation	slight callus formation
NAA 1 mg/l	no response	not tested	not tested
IAA[e] 1 mg/l	no response	no response	no response
BAP 1 mg/l	shooting	no response	no response

[a]Naphthalene acetic acid.
[b]6-Benzylaminopurine.
[c]2,4-Dichlorophenoxyacetic acid.
[d]Kinetin.
[e]Indole-3-acetic acid.

Previously, Page and Van Staden (12) reported that, in the case of *H. rooperi*, root differentiation and meristematic bulging were the major proliferative responses of corm explants to auxin (NAA) stimulation. On the contrary, in our experiments callus proliferation was rarely observed on auxin-containing media (NAA or 2,4D), and it was usually confined to the cut margins of the explants. These small callus formations failed to proliferate when transferred from the original explants to fresh medium. Furthermore, root differentiation was never observed either from the original corm explants or from the in vitro proliferating tissues (shoots and/or calli). These findings, together with the negative results obtained with *H. nyasica* and *H. angustifolia*, confirm the strong influence of the genetic background of the test plant on its in vitro behavior.

On the other hand, in agreement with the results of Page and Van Staden (12), we found that shoot generation was easily obtained by culturing the explants on

culture and were able to root spontaneously, either on the same culture medium or after transfer to MSO (¼ strength).

Regenerated plantlets were kept in culture under aseptic conditions and gave rise to rhizome formation after a period of 6 to 10 months. These plantlets were utilized as starting material to determine the content and distribution of the active glucoside (Table 2).

HYPOXOSIDE CONTENT.—The analysis of *H. obtusa* plants showed a prevalent localization of hypoxoside in the inner part of the rhizome. The content decreased considerably in the bark and drastically dropped in the roots (Table 2). The value obtained for the whole rhizome (5.37%) appeared higher than that previously determined by means of counter-current distribution (3.7%) (3) and that reported for *H. rooperi* analysis (3.5–4.5%), which, however, was based on a different procedure (4).

Two main factors must be taken in account in order to assess the significance

TABLE 2.   Hypoxoside Content in Organs of Parental and
Regenerated Plants of *Hypoxis obtusa*.

Organ	Hypoxoside content (% of fresh mass)	
	Parental	Regenerated
Rhizome bark	3.13 ± 0.01	—
Inner rhizome	6.37 ± 0.08	—
Total rhizome	5.36 ± 0.02	0.013 ± 0.01^a
Roots	0.74 ± 0.02	0.002 ± 0.0002
Leaves	not detectable	not detectable

[a]Values determined after 8 weeks of culture.

of content determination: the presence in the EtOH extract of a great quantity of interfering polar substances and the difficulty of separation of hypoxoside from nyasoside and related substances, which presented similar distribution although with much lower concentration in comparison with hypoxoside. The first problem was overcome by a preliminary partitioning between H_2O and n-BuOH and the second problem by subsequent purification by the use of a weakly acid buffer in the chromatography.

The same analysis carried out on the corresponding organs of regenerated plants showed an analogous distribution of hypoxoside, albeit at considerably lower levels. However, as observed by Page and Van Staden in the case of *H. rooperi* (13), it is possible that the content of hypoxoside increases as a function of rhizome age.

As an alternative to this approach, work is in progress in our laboratory to test the possibility that cultures of roots induced by infection with the hairy root organism (*Agrobacterium rhizogenes*) might constitute a more suitable material for the large scale preparation of hypoxoside (14).

EXPERIMENTAL

PLANT MATERIAL AND TISSUE CULTURE.— *H. obtusa* plants (obtained from the Instituto Nacional de Investigaciones Agronomicas, Maputo, Mozambique, where the species was determined by Mr. L. Macuacua) were cultivated at the Botanical Garden of the University of Rome, where voucher samples remain. *H. nyasica* was collected near Zomba, Malawi, and *H. angustifolia* near Antanarivo, Madagascar; the species were identified at the local universities and cultivated in Rome.

For tissue culture, corms were washed exhaustively with H_2O, incubated in a 5% commercial bleach solution for 5 min, and washed in running tap H_2O. The outer tissues of the corms were removed with a knife and the inner tissues further treated with a 5% saline HClO solution for 20 min. Corms were carefully washed in sterile tap H_2O (5 to 8 changes), cut into small pieces (about 1 cm thick), and plated onto different hormone-containing culture media. The basic nutrient medium of Murashige and Skoog (MS) (11) (Flow Laboratories) was utilized with addition of 30 g/liter sucrose, 1 g/liter vitamin-free casein hydrolysate, 0.8% Difco Bacto-agar, and 1 mg/liter thiamine HCl, as specified by Page and Van Stade (12).

Various concentrations of 6-benzylaminopurine (BAP), 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4D), indole-3-acetic acid (IAA), and kinetin were used, as indicated in Table 1. The pH was adjusted to 6.0 before autoclaving (20 min, 120°). Five replicates were prepared for each hormone treatment. Explants were incubated in a growth chamber (25°, 16 h illumination period, 8000 lux) and scored for morphogenetic response after 3–4 weeks. Plantlets regenerated from the corm explants were kept in aseptic culture on MS medium (¼ strength) and transferred every 6–8 weeks. Calli originating from the cut margins of the explants were subcultured on the medium containing the same hormone combination and transferred every 3–4 months.

ANALYSIS OF GLUCOSIDE CONTENT.—Plant material from *H. obtusa* (10 g for each analysis) was homogenized to a mush and extracted with MeOH at room temperature. After evaporation of the solvent in vacuo, the residue was dissolved in H_2O (20 ml) and the solution extracted with n-BuOH saturated H_2O (4 × 10 ml).

The combined organic extract was concentrated to 10 ml, and 0.5 ml was added to 2 ml of an aqueous solution of HOAc (1:250) containing a few drops of MeOH. The solution was poured onto a Sep-Pak C-18 cartridge. The cartridge was first washed with 5 ml of a mixture of HOAc (1:250) and MeOH (7:3) for the elution of more polar substances, whereas the glucoside fraction was eluted with 5 ml of MeOH-HOAc (1:1). The second fraction was evaporated, the residue dissolved in MeOH (0.5 ml), and the solution analyzed.

The hplc system consisted of a Model LC Pump Series 410, a spectrophotometric detector LC 90, and a Computing Integrator LCI-100, all from Perkin-Elmer. A reversed-phase μ-Bondapak C-18 column (30 cm × 3.9 mm i.d.) (Water Associates, Milford, Massachusetts) was used. The gradient elution was performed at 25° with mobile phases A and B, consisting of 0.05 M NaOAc buffer (pH 3.5) for A and MeOH for B. The gradient program started at sample injection and was linear from 60% A and 40% B to 20% A and 80% B over 30 min. The flow rate was 1.0 ml/min, and the wavelength chosen (260 nm) was optimum for analytic sensitivity and minimal gradient background.

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