STUDIES ON ALKALOIDS OF REED CANARYGRASS

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

Bruce Edward Coulman

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosphy

Department of Plant Science

August 1976

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by

BRUCE EDWARD COULMAN

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ACKNOWLEDGMENTS

Many people made contributions that in some way aided in the completion of this Ph.D. program. The author would like to acknowledge certain people whose contributions to this research and thesis were substantial.

The author would like to express sincere thanks to Dr. K. W. Clark for suggesting the topic of this research and for his helpful suggestions and encouragement during the course of the work.

Special thanks are due to Dr. D. L. Woods for his invaluable advice on laboratory procedures.

Gratitude is expressed to Dr. A. K. Storgaard and Dr. W. Woodbury for careful review of the manuscripts, to Dr. J. R. Ingalls for advice on the in vitro digestion study and to Mr. Allen Cox of the Manitoba Department of Agriculture for acid detergent fiber analyses.

Last, but not least, the author would like to thank his wife, Lorraine, for continuous encouragement and for careful typing of the thesis.

Financial support from the National Research Council and Canada Department of Agriculture was greatly appreciated.

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ABSTRACT

Coulman, Bruce Edward. Ph.D. The University of Manitoba, August, 1976. Studies on Alkaloids of Reed Canarygrass. Major Professor: K. W. Clark.

Fifty-two strains of reed canarygrass were screened for tryptamines and carbolines and for concentrations of gramine. Most strains contained genotypes free of tryptamines and carbolines and showed wide interplant variation in gramine.

A colorimetric assay for the determination of hordenine concentrations in reed canarygrass was developed. Screening of several diverse genotypes showed a wide variation for this alkaloid. Many genotypes contained higher concentrations of hordenine than gramine. Data for both gramine and hordenine were highly correlated between years and sampling times, indicating high broad sense heritability. Narrow sense heritability estimates of 0.72 for gramine and 0.53 for hordenine concentrations were determined. It was concluded that there exists a diverse gene pool from which to select tryptamine-carboline free, low gramine, low hordenine strains of reed canarygrass.

The distribution within the plant and variation with maturity of gramine and hordenine concentrations were also examined. Concentrations of gramine were highest in leaf blades of reed canarygrass, while hordenine was most concentrated in the leaf sheath.

Concentrations of both alkaloids in leaf blades were highly correlated to the concentrations in the total forage. In first growth reed canarygrass, concentrations of gramine and hordenine in leaf blades increased slightly from a vegetative stage to early heading. In young regrowth, concentrations of both alkaloids increased considerably over those of first growth forage, but in more mature regrowth, had declined to levels found in first growth.

Another study was undertaken to determine the effect of selected reed canarygrass alkaloids on in vitro digestibility. A range of concentrations of gramine, hordenine, N-methyl tryptamine and 5-methoxy-dimethyl tryptamine were added to in vitro rumen fluid fermentations. No relationship was found between concentrations of these alkaloids and in vitro digestion values. As well, nor were alkaloid concentrations of selected reed canarygrass clones related to their in vitro digestibility.

FORWARD

This thesis has been prepared in manuscript format. It consists of a literature review, three manuscripts prepared as recommended by the Canadian Journal of Plant Science, and a general discussion. Manuscript I, "Identification of Low Alkaloid Genotypes of Reed Canarygrass", has been accepted for publication by the Canadian Journal of Plant Science. Manuscript II, "The Effect of Selected Reed Canarygrass Alkaloids on In Vitro Digestibility", and Manuscript III, "Distribution Within the Plant, Variation with Maturity, and Heritability of Gramine and Hordenine in Reed Canarygrass", will be submitted to the Canadian Journal of Plant Science.

INTRODUCTION

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Reed canarygrass (Phalaris arundinacea L.) is a forage grass with considerable potential. Most studies (Wilkins and Hughes, 1932; Wedin et al., 1966) have shown it to be among the highest yielding of the cool season forage grasses grown in North America. It is also very responsive to nitrogen fertilization (Niehaus, 1971; Dean and Clark, 1972). In general, on poorly drained, wet areas, reed canarygrass is the most productive and persistent grass available. There are large areas of wetland in Manitoba, the North-Central United States and Eastern Canada. Due to two major problems, however, reed canarygrass has not become the leading forage grass of these areas.

Seed of reed canarygrass tends to shatter readily upon ripening. This has caused reed canarygrass seed to be more expensive than seed of other cool season grasses and often unavailable. The recent development of a non-shattering cultivar, Castor, should help to overcome this problem.

The most frequently cited reason why reed canarygrass has not become a leading forage grass is lack of palatability (Marten and Heath, 1973). When reed canarygrass is grown with other cool season frasses, animals will usually consume more of the other species. As well, poor weight gains (Van Arsdell et al., 1954; Marten and Jordan, 1974; Marten et al., 1976) and evidences of toxicity (Van

Arsdell et al., 1954; Parmer and Brink, 1976) have been seen in animals grazing reed canarygrass.

Over the past 20 years, 9 alkaloids (gramine, hordenine, 4 tryptamine derivatives and 3 β-carbolines) have been identified in reed canarygrass (Marten, 1973; Gander et al., 1976). This has led to a great deal of research into factors affecting the concentration of these compounds, their inheritance, and their relationship to the poor animal performance occurring with this grass. The total alkaloid content of reed canarygrass has been found to be negatively correlated with palatability (Simons and Marten, 1971) and average daily gains of cattle and sheep (Marten et al., 1976). Woods and Clark (1971a) found that the presence of tryptamines was controlled by a single dominant gene, while Barker and Hovin (1974) reported high narrow sense heritability estimates for total alkaloid contents.

The program at the University of Manitoba has tended to focus on the individual alkaloids of reed canarygrass. Its ultimate goal is to produce tryptamine free strains of reed canarygrass that are low in gramine and hordenine. To aid in achieving this goal, the present study was undertaken to: 1) Determine simple and rapid techniques for the extraction and estimation of hordenine, 2) Determine the variability and heritability of concentrations of gramine and hordenine, 3) Determine the distribution within the plant and the variation with plant maturity of gramine and hordenine.

The present study also examines the effect of the alkaloids of

reed canarygrass on *in vitro* digestiblity. This research was suggested by the work of Bush *et al.* (1970, 1972) who found that perloline, the major alkaloid of tall fescue (*Festuca arundinacea* Schreb.), had a marked inhibitory effect on *in vitro* digestion. This work raises the possibility that poor animal performance may be due to alkaloidal interference with the rumen microflora.

LITERATURE REVIEW

LITERATURE REVIEW

Description and Adaptation of Reed Canarygrass

Reed canarygrass (Phalaris arundinacea L.) is a long-lived, cool season perennial. It grows in dense clumps, but will spread by rhizomes to form a dense sod if well managed. Flowers are borne on semi-dense panicles which contract following anthesis. Seeds mature from the top of the panicle downward and shatter readily at ripening.

Native stands of reed canarygrass are found in temperate regions of North and South America, Europe, Asia and Africa. It is well adapted to the northern United States and most agricultural regions of Canada. Although its natural habitat is poorly drained, wet areas, it has proven to be quite drought tolerant on upland soils. It tends, however, to winter-kill on upland prairie soils if snow cover is sparse. Seeds, seedlings and mature plants show remarkable tolerance to flooding.

Genetics

Reed canarygrass is most commonly a tetraploid (2n=28) (Darlington and Wylie, 1955). McWilliam and Neal-Smith (1962) screened a wide range of introductions and found 2 chromosome races, one tetraploid and the other hexaploid (2n=42). The majority of the hexaploids were from Spain and Portugal, whereas the tetraploids came from more northerly countries. Strains grown in North America are likely

tetraploid although Superior, a variety of obscure origin developed in Oregon, is a hexaploid. Diploids (2n=14) have also been reported (Poehlman, 1959). Woods (1971) found seedlings of the variety Grove to be tetraploid. Both the hexaploid and tetraploid behave as allopolyploids with regular meiosis and stable chromosome number (McWilliam and Neal-Smith, 1962). There is, of course, the possibility of gene duplication in these polyploids. Single dominant genes have been shown, however, to control the presence of an anthocyanin pigment (McWilliam and Shepherd, 1964) and the presence of tryptamine alkaloids (Woods and Clark, 1971a).

Agronomic Potential

Studies throughout Canada and the United States have shown reed canarygrass to be a very productive hay species. Early work reported hay yields of 9-20 t/ha in the United States (Schoth, 1929). Reed canarygrass was the most drought resistant and highest yielding of seven cool-season grasses in tests conducted 1925-1928 (Wilkins and Hughes, 1932). More recent studies in the North-Central United States have reported yields of 13-17 t/ha with proper fertilization and management (Niehaus, 1971; Marten and Heath, 1973). Extensive testing in Western Canada (Tingle and Elliott, 1974) has shown reed canarygrass yields to be comparable to those of timothy (Phleum pratense L.), intermediate wheatgrass (Agropyron intermedium (Host.) Beauv.) and bromegrass (Bromus inermis Leyss.). In Eastern Canada, however, yields are consistently poorer than those of timothy on upland soils (Goplen et al., 1963). On land that is poorly drained and subject to flooding, reed canarygrass is the most persistent and productive

grass species available (Goplen et al., 1963).

Hay yields of reed canarygrass have proven to be very responsive to nitrogen fertilization. Linear yield increases with increasing N up to 336 Kg/ha (Dean and Clark, 1972) and increases up to 600 Kg N/ha (Niehaus, 1971) have been reported.

For grazing purposes, reed canarygrass is one of the highest yielding perennial grasses in its area of adaptation (Marten, 1973). It has consistently outyielded bromegrass in pasture trials in the North-Central United States (Wedin et al., 1966; Marten and Donker, 1968). It grows vigorously in the spring and will regrow well throughout the summer and fall if moisture is adequate (Goplen et al., 1963).

Reed canarygrass, because of its vigorous spreading growth, has proven useful in prevention of soil erosion. For healing and controlling gullies, it is unexcelled (Hughes et al., 1952) and it is frequently used for maintenance of grass waterways, stream channel banks and edges of farm ponds (Marten, 1973).

More widespread utilization of this species has been hindered by erratic seed production caused by the seed shattering habit. Vose (1959) reported North American seed yields vary from 56-560 Kg/ha with the average being 56-168 Kg/ha. Wide variability in seed retention has been found (Baltensperger and Kalton, 1959) and it has been shown to be a highly heritable character (Bonin and Goplen, 1966). Recently a seed retaining variety, Castor, was licensed in Canada (Canada Dept. Agr. Plant Products Div. #1413).

QUALITY CHARACTERISTICS

Chemical Composition

Reed canarygrass compares favourably with most other forage species in crude protein (CP) content. Goplen et al. (1963) reported CP contents at the pasture stage ranged from 20-27%. With an application of 600 Kg N/ha per year, CP levels averaged 19.2% over three cuts (Niehaus, 1971). CP percentages similar to those found in alfalfa at a similar growth stage have been reported (Barnes and Mott, 1970). Average CP percentages of reed canarygrass were significantly greater than percentages found in bromegrass and intermediate wheatgrass (Lawrence et al., 1971), but levels in reed canarygrass hay were lower than in timothy and redtop (Agrostis alba L.) hay (Schoth, 1929). Reed canarygrass was among the highest in CP of 15 grasses sampled at early heading (Tingle and Elliott, 1975).

Reed canarygrass has been shown to contain crude fiber percentages similar to those found in timothy and redtop (Schoth, 1929). It is similar to alfalfa in lignin percentage at early bloom (O'Donovan et al., 1967b). In neutral detergent fiber content, reed canarygrass was lower than brome at the first cut, but higher than brome, alfalfa and trefoil (Lotus corniculatus L.) in aftermath growth (Ingalls et al., 1965).

Digestibility

Ingalls et al. (1965) found reed canarygrass to be as digestible as alfalfa at the second and third cuts in a season but less digestible at the first. A number of workers have reported reed canarygrass to be equal to or greater than other cool season perennial grasses in digestibility (Pritchard et al., 1963; Pringle and Miltimore, 1966).

A more recent study showed, however, that reed canarygrass was among the least digestible of 15 grasses sampled at early heading (Tingle and Elliott, 1975). Due to its dormancy in late fall reed canarygrass loses digestibility more rapidly than an active fall growing species such as tall fescue (Festuca arundinacea Schreb.) (Wedin et al., 1966).

Palatability, Intake and Animal Performance

Poor palatability is the most often cited reason that reed canarygrass has not become a major species (Marten, 1973), but studies on the palatability of this species have produced conflicting results. Schoth (1929) reported reed canarygrass hay as being less palatable than other grass hays, while Rogler (1944) found reed canarygrass to be the least palatable of 10 cool season grasses at all times during the grazing season. Marten and Donker (1968) showed that dairy heifers distinctly preferred bromegrass to reed canarygrass, and Marten and Jordan (1974) reported similar results with sheep. Wilkins and Hughes (1932) found, however, that reed canarygrass was equal or better in palatability than all cool season grasses tested except Canada bluegrass (Poa compressa L.), bromegrass and timothy. Goplen et al. (1963) reported high palatability in reed canarygrass pasture and hay if grazed or cut before full heading.

Significant genetic variability for palatability among reed canary genotypes has been found (Asay et al., 1968). Palatability was also shown to be moderately heritable in reed canarygrass (Asay et al., 1968; Barnes et al., 1970). Correlations of palatability between regrowths of the same material have, however, been quite

variable (Barnes et al., 1970; Woods and Clark, 1974). Roe and Mottershead (1962) found hexaploid genotypes to be much less palatable than tetraploids.

In evaluating the significance of palatability one must look at intake and subsequent performance of animals grazing one species without access to another. Van Arsdell et al.(1954) found poor and inconsistent gains in sheep grazing reed canarygrass. Switching to bluegrass or alfalfa resulted in immediate greater gains. Intakes for reed canarygrass by sheep were considerably lower than for alfalfa or trefoil, but similar to bromegrass. Marten and Donker (1968) showed that although heifers preferred bromegrass to reed canarygrass, daily gains were equal on the two species when they were grazed without choice. In the case of sheep, however, both palatability and weight gains with reed canarygrass were lower than with orchardgrass (Dactylis glomerata L.) or bromegrass (Marten and Jordan, 1974). Other studies have reported weight gains on reed canarygrass comparable to gains on other grasses (Hubbard and Nicholson, 1968; Thomas et al., 1965). Thus, there has been conflicting data on the performance of reed canarygrass as compared to other grasses. In general, there have been more reports of poor performance with sheep than with cattle.

Studies have also been done on genotypes of reed canarygrass differing in palatability. O'Donovan et al.(1967a) found organic matter intake of two palatable clones to be greater than the intake of two unpalatable clones in all grazing experiments. Using the same clones in four feeding trials with sheep, Barnes and Mott (1970)

found, however, that the intake of the unpalatable clones was greater in one trial.

Toxicity

Incidences of reed canarygrass toxicity to grazing animals have been found. Van Arsdell et al. (1954) reported rough haircoats and profuse eye watering of steers grazing reed canarygrass pastures in Michigan. Audette et al. (1970) examined the livers of cattle performing poorly on reed canarygrass pastures in Manitoba and found lesions, which they described as "alkaloid-type". Severe diarrhea has been seen in sheep and cattle grazing experimental reed canarygrass pastures (Woods and Clark, 1974; Marten et al., 1976). A high incidence of bovine pulmonary emphysema has been found in British Columbia on wetland meadows in which reed canarygrass was a major species (Parmer and Brink, 1976).

More severe disorders have been seen in animals grazing hardinggrass (Phalaris aquatica L.), a species closely related to reed canarygrass. Sheep grazing hardinggrass pastures in Australia often suffer from "phalaris staggers" or "sudden death" (Gallagher et al., 1964, 1966). Phalaris staggers may take an acute form from which recovery is not possible or may terminate in a chronic form involving degeneration of the central nervous system. Symptoms of both forms include incoordination, crippling and hyperexcitability. Sudden death is the term applied to a peracute disease involving sudden collapse, followed by death from cardiac arrest or by apparent complete recovery. Gallagher et al. (1966) reported that the chronic form of the staggers disease could be overcome by supplementation of

the diet with cobalt. Cobalt had no effect on the acute staggers or sudden death.

Alkaloids of Reed Canarygrass

Alkaloids are a group of compounds that have the following properties: 1) chemically basic; 2) nitrogen containing; 3) of plant origin; 4) significant pharmacological activity; and 5) complex molecular structure (Pelletier, 1970). Robinson (1974) states that alkaloids should no longer be considered to be only of plant origin since animals produce compounds that have all the characteristics of alkaloids. Several authorities refer to simple amines as "protoalkaloids", reserving the name alkaloids for more complex compounds with nitrogen in a ring.

About 2,000 alkaloids have been identified and they are present in 10-15% of all vascular plants (Pelletier, 1970). A review by Robinson (1974) concludes that although alkaloids appear to be active metabolites, their usefulness to plants remains obscure.

Occurrence

Reports of poor animal performance with reed canarygrass led workers to investigate the chemical consituents of this species. Wilkinson (1958) isolated 5 methoxy,N-methyl tryptamine (5MeO-NMT) and hordenine from reed canarygrass. Culvenor et al.(1964) found gramine to be the major alkaloid of several unpalatable strains. The presence of 5 methoxy,N,N-dimethyl tryptamine (5MeO-DMT) and dimethyl tryptamine (DMT) was reported by several workers in the same year (Woods and Clark, 1971b; Williams et al., 1971; Barnes et al., 1971). Woods and Clark (1971b) also identified N-methyl

tryptamine (NMT) in reed canarygrass. Three β-carbolines have also been identified: 2,9 dimethyl-6-methoxy-1,2,3,4,tetrahydro-β-carboline (Audette et al., 1969, 1970); 2-methyl-6-methoxy-tetrahydro-β-carboline (Marten, 1973); and 2-methyl,1,2,3,4,tetrahydro-β-carboline (Gander et al., 1976). Structures of the reed canarygrass alkaloids are given in Appendix 1.

There have been no reports of NMT, 5MeO-NMT and the dimethylated β-carboline occurring in hardingrass. This species contains the other six alkaloids found in reed canarygrass and also bufotenine (5 hydroxy,N,N-dimethyl tryptamine).

Hordenine occurs in several other grasses and in many dicotyledonous species (Culvenor, 1973). Gramine is found in the grass species, Hordeum vulgare and Arundo donax. The latter species also contains DMT and 5MeO-NMT. Gramine and the tryptamines are also widespread in the dicots.

Biosynthesis

Studies with barley (Hordeum vulgare) have shown that hordenine may be derived from tyrosine or phenylalanine (Robinson, 1968). A precursor of hordenine, tyramine, is formed by decarboxylation of tyrosine or by decarboxylation and hydroxylation of phenylalanine. Tyramine is then N-methylated, the methyl groups being derived from methionine.

Gramine, tryptamines and β-carbolines are derived from tryptophan. Tryptophan decarboxylase has been partially purified from P. aquatica (Baxter and Slaytor, 1972a). Further work by Baxter and Slaytor (1972b) indicated that at least five pathways could be operating in biosynthesis

of 5MeO-DMT from tryptophan. These pathways involved various methylations and methoxylations of tryptamine. The β-carbolines can be readily synthesized *in vitro* by reaction of tryptamine with aldehydes (Robinson, 1968). The α-carbon of the aldehyde forms the new ring by joining the amino nitrogen to the existing ring.

Gramine is peculiar in that it has a shorter side chain than tryptamine. It has been shown, however, to be derived from tryptophan in barley seedlings (O'Donovan and Leete, 1963) and its immediate precursors are 3-aminomethylindole and 3-methyl-aminomethylindole (Mudd, 1969; Gower and Leete, 1963).

Extraction, Purification, Characterization and Quantitative Estimation

Alkaloids can be extracted by making plant material alkaline and extracting the free bases into an organic solvent. Extracts can then be purified by partitioning into acid, basifying, and re-extracting into an organic solvent (Robinson, 1963). This leaves neutral and acidic water soluble compounds behind. Most methods used to extract and purify reed canarygrass alkaloids follow this general procedure. Culvenor *et al.*(1964) extracted macerated samples with methanol in a Soxhlet apparatus and purified by the acid base partitioning procedure. Woods and Clark (1971a) eliminated the Soxhlet extraction by standing chopped samples overnight in a mixture of chloroform, methanol and ammonia. Frelich and Marten (1973) prepared plant extracts by macerating frozen grass in ammoniacal chloroform and partitioning into 2N H_2SO_4. This acid extract was used for further analyses.

Culvenor *et al.*(1964) used paper chromatography to separate the

alkaloids in crude extracts from hardinggrass. Erlich's reagent or α-nitroso-β-naphthol reagent were used to detect the alkaloids on the chromatograms. Woods and Clark (1971b) separated reed canarygrass alkaloids by thin-layer chromatography and detected them with Erlich's or xanthydrol reagent. Gas chromatography has also proven useful for separating and characterizing reed canary alkaloids (Audette et al., 1969, 1970; Williams et al., 1971).

Colorimetric assays for estimation of alkaloid concentration have been developed. The basis of several assays has been the finding of Dickman and Crockett (1956) that the reaction between xanthydrol and tryptophan in acid solution forms a highly coloured stable product. Moore et al.(1967) and McComb et al.(1969) removed coloured spots from paper chromatograms sprayed with xanthydrol, and dissolved them in HCl for colorimetric determination. Woods and Clark (1972) reported a colorimetric assay for gramine involving no prior chromatographic separation. This procedure could only be done with material free of tryptamines and carbolines. Frelich and Marten (1973) developed a rapid test for determination of total alkaloid content in reed canarygrass. This procedure involved visual assessment of precipitates produced by silicotungstic acid added to plant extracts.

Environment Influence

Environment has been shown to have an effect of the alkaloid content of hardinggrass. Levels of tryptamine alkaloids increased with shading, increased nitrate levels and higher temperatures (Moore et al., 1966, 1967). Williams (1972) found alkaloid concentrations

varied with time of day but the pattern was not consistent from day to day. This study also showed that severe wilting and frosting caused increases in alkaloid concentrations.

In controlled studies at the University of Minnesota, shading of reed canarygrass plants caused 25–50% increases in total alkaloid content (Marten, 1973). Moisture stress more than doubled alkaloid contents, while temperature and photoperiod had only slight effects. Marten et al.(1974) examined the effect of nutrient supply on alkaloid levels. The alkaloid concentration of reed canarygrass grown on infertile peat soils was reduced when deficient nutrients were supplied. On fertile mineral soils, low levels of added nitrogen (60 Kg/ha) increased alkaloid levels in high alkaloid genotypes. In low alkaloid genotypes, relatively high levels of added nitrogen (at least 200 Kg/ha) were required to increase alkaloid contents. Ammonium sources of nitrogen caused greater alkaloid concentrations than nitrate sources. This supports the hypothesis of James (1950) that NH_4-N is probably used directly for alkaloid synthesis by plants, whereas NO_3-N is more functional for overall growth.

Distribution Within the Plant

According to Robinson (1974), alkaloids generally accumulate in the most metabolically active part of the plant. This appears to be the situation in reed canarygrass. Hagman et al.(1975) found leaf blades to have twice the alkaloid content of stems and sheaths combined. Roots and rhizomes were found to contain 0.01% alkaloid by dry weight when forage contained 0.19% (Marten, 1973). Parmar

and Brink (1976) found stems and sheaths to be free of tryptamines, with the highest concentrations occurring in the youngest leaves. Thus, indole alkaloids of reed canarygrass are probably synthesized in leaf blades. This is in accordance with the finding of Bowden and Marion (1951) that gramine is derived from tryptophan in barley leaves.

Effect of Plant Maturity and Cutting Management

Woods and Clark (1971a) found that gramine and tryptamine contents (fresh weight basis) of first growth reed canarygrass rose to a maximum at about the time of seed shattering. The levels in regularly clipped grass continued to increase into the fall and were substantially higher than in unclipped samples. Marten (1973), however, reported a 40% decline in alkaloid content (dry weight basis) of unclipped grass between a vegetative stage and anthesis. Marten suggested that the results of Woods and Clark for unclipped grass would have been similar had they been expressed on a dry weight basis. Frelich and Marten (1972) found the alkaloid concentration of 5-week regrowth to be less than half of that in 2-week regrowth. Hagman et al.(1974) found alkaloid contents of regrowth herbage to be significantly higher than in first growth and that levels of both regrowth and first growth herbage declined with maturity. Parmer and Brink (1976) reported similar declines for tryptamine levels of reed canarygrass pastures in British Columbia.

Variation and Inheritance

In hardinggrass, there are substantial differences (0.05% - 0.178% DW)

in alkaloid content among strains and introductions (Oram and Williams, 1967; Oram, 1970). Rendig et al. (1970) reported wide variations in tryptamine contents of individual plants of this species.

Variation in total alkaloid content among reed canarygrass strains was not as large. In an analysis of six strains including the licensed varieties Rise, Grove, Frontier, and Vantage, the 2-year means ranged from 0.118% to 0.170% (Hovin and Marten, 1975). Another study by Marten (1973) showed that mean alkaloid contents of six strains ranged from 0.36% to 0.46%. The higher levels in the latter study were partially due to regrowth material being sampled whereas the former was an average of regrowth and first growth samples.

Individual genotypes of reed canarygrass showed a much wider range in alkaloid content than did varieties. Ranges in total alkaloid content (% Dry Weight) among genotypes that have been reported include: 0.01 to 2.75% (Simons and Marten, 1971); 0.20 to 0.91% (Williams et al., 1971); and 0.15 to 1.19% (Marten et al., 1973). Marten (1973) reports, however, that levels greater than 1.0% DW occur only rarely and that distributions within the reported ranges are skewed towards lower concentrations.

Woods and Clark (1971b) examined the alkaloid types of individual clones of reed canarygrass. They found that approximately half of the clones grown from seed of two Ottawa synthetics were entirely free of tryptamines. These tryptamine free plants contained gramine and hordenine but were apparently free of β-carbolines. The presence of tryptamines was found to be controlled by a single dominant gene (Woods and Clark, 1971a). Thus, removing tryptamine containing

genotypes from a breeding nursery would eliminate this character from the population.

Marten et al.(1973) grew a number of clones in three North-Central States and in Alaska. They found total alkaloid content of the clones to be highly correlated between all environments (location, harvest, and year variables). As well, the primary alkaloid types of each clone were very repeatable among locations. These workers suggested that although breeders can accurately screen for alkaloid differences in a single location, all plants should be harvested at a similar growth stage. This is necessary since absolute alkaloid concentrations vary greatly with maturity.

Barker and Hovin (1974) found variances for genotype x environment and genotype x cutting interactions to be small as compared to genetic variance for alkaloid content. They also found high narrow sense heritabilities (h^2 = 0.55 - 0.78 for combined results from two cuttings) for total alkaloid content. Narrow-sense estimates were almost as high as corresponding broad-sense estimates, indicating high additive genetic variance. Oram (1970) reported the concentration of tryptamines in hardinggrass was greatly influenced by environment, but heritability in any given environment was high (h^2 = 0.92 in one study). He concluded that selection for low tryptamine levels would be effective.

Relationship to Quality

Palatability, Intake and Performance. First evidence for an unpalatable factor in reed canarygrass was given by Roe and Mottershead (1962). Extraction of unpalatable strains with organic solvents made

these strains more palatable. As well, when extracts of unpalatable strains were sprayed on palatable strains, the latter became unpalatable. This study perhaps points to the alkaloids as the unpalatable factor.

Simons and Marten (1971) found a high correlation between palatability (1 consumed to 10 rejected) and total alkaloid content of a number of diverse reed canarygrass genotypes. Clones with an alkaloid content greater than 0.8% dry weight were almost totally rejected while those with less than 0.2% were readily consumed. The primary alkaloid type (gramine, DMT, 5MeO-DMT) was not associated with palatability. Barnes et al.(1971) and Williams et al.(1971) found, however, that unpalatable clones contained much higher levels of 5MeO-DMT than palatable clones. Palatable clones were also free of DMT. These results must be interpreted with caution, however, as only four clones were used.

Jordan and Marten (1975) found reduced weight gains in ponies grazing reed canarygrass pastures from late July to mid September when alkaloid contents were highest (0.32% DW). Earlier in the season with lower levels (0.10% DW), gains were better than with bromegrass and orchardgrass (Dactylis glomerata L.). Woods and Clark (1974) found weight gains of sheep to be poor on both tryptamine containing and tryptamine free pastures. Donker et al.(1976) reported weight losses in lambs fed fresh reed canarygrass while those fed reed canarygrass hay showed substantial weight gains. The fresh grass contained 69% more alkaloids than the hay.

Work by Marten et al.(1976) demonstrated the relationship

between alkaloids, intakes, and animal performance. Using clonal material, they established four pastures, two consisting of tryptamine-carboline free genotypes (one high in total alkaloids and one low) and the other two consisting of tryptamine-carboline containing genotypes (one high in total alkaloids and one low). Both lambs and steers grazed these pastures in separate experiments. With lambs, the high alkaloid pastures were less readily consumed and the weight gains were less than on the low alkaloid pastures. With steers, there were no differences in intake among the pastures, but weight gains were poorer on the high alkaloid pastures. In general, between the pastures with similar alkaloid levels, gains were poorer on those containing tryptamines and carbolines. Both lambs and steers showed a higher incidence of diarrhea on high alkaloid pastures and especially on those containing tryptamines and carbolines. Thus, it appears that a physiological disturbance, as evidenced by the diarrhea, may contribute to the reduced gains in lambs and steers. In the case of lambs, poor consumption of high alkaloid pastures also affects weight gains.

Digestibility. Studies have also been done to determine whether alkaloids can affect animal performance by interfering with the rumen microflora. Tall fescue is an alkaloid containing species, with perloline being one of its major alkaloids (Tookey and Yates, 1972). Bush et al. (1970) found that perloline inhibited in vitro cellulose digestion by microorganisms. Further work by Bush et al. (1972) showed a stimulation of volatile fatty acid production at low concentrations of perloline and an inhibition at high concentrations. Growth of

rumen bacteria was completely inhibited at these higher concentrations.

Marten (1973) found no relationship in alkaloid content of reed canarygrass forage and in vitro digestible dry matter (IVDDM). Addition of pure samples of gramine and the dimethyl tryptamines to various forages had little effect on IVDDM. Extremely high concentrations of 5MeO-DMT (3.0% DW) did, however, significantly depress IVDDM. Work in Australia showed that addition of gramine to orchardgrass caused a substantial decrease in in vitro digestibility (G.W. Arnold, CSIRO, Wembley, Western Australia, personal communication).

Toxicity. Gallagher et al.(1964, 1966) injected sheep with pure samples of the dimethyl tryptamines found in hardinggrass. They found that the symptoms of the acute phalaris staggers and the peracute sudden death diseases were duplicated by these injections. The most potent alkaloid in producing these disorders was 5MeO-DMT. These workers suggested that the dimethylated tryptamines interfere with the normal pharmacological functions of serotonin (5-hydroxy-tryptamine) in the mammalian system. These functions include: 1) smooth muscle contraction; 2) cardiac activity; 3) normal functioning of the brain; and 4) probably a chemical transmitter in the central nervous system. As well, tryptamines and β-carbolines are potent inhibitors of monamine oxidase, the enzyme responsible for controlling levels of serotonin and other amines in the central nervous system (Gallagher et al., 1966; Ho et al., 1968). Thus, the alkaloids of hardinggrass have been directly related to the toxicities occurring with this species.

In reed canarygrass, a higher incidence of diarrhea has been reported on tryptamine-containing pastures than on tryptamine-free pastures (Woods and Clark, 1974; Marten et al., 1976). Parmer and Brink (1976) related the incidence of bovine pulmonary emphysema to high tryptamine levels in pasture regrowth following a hay cut.

MANUSCRIPTS

MANUSCRIPT I

IDENTIFICATION OF LOW ALKALOID GENOTYPES OF REED CANARYGRASS

IDENTIFICATION OF LOW ALKALOID GENOTYPES OF REED CANARYGRASS

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ABSTRACT

Fifty-two strains of reed canarygrass (Phalaris arundinacea L.) were screened for the presence of tryptamines and carbolines and for concentrations of gramine. Most strains contained genotypes free of tryptamines and carbolines and showed wide interplant variation in gramine levels. Gramine data between years were highly correlated indicating high broad sense heritability. It was concluded that there exists a diverse gene pool from which to select tryptamine-carboline-free, low-gramine strains of reed canarygrass. An improved method for the determination of gramine concentration is described.

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INTRODUCTION

Reed canarygrass (Phalaris arundinacea L.) has considerable potential as a forage crop (Vose, 1959; Marten and Heath, 1973). There have, however, been reports of poor palatability (Rogler, 1944; Marten and Donker, 1968) and poor animal performance (Van Arsdell et al., 1954) with this species when compared to other cool season grasses. Eight alkaloids (gramine, hordenine, four tryptamine derivatives and two β-carbolines) have been reported in reed canarygrass (Marten, 1973). Barker and Hovin (1974) found a high heritability for total alkaloid concentration, while Woods and Clark (1971a) showed the presence of tryptamines to be controlled by a single dominant gene. Further studies showed that tryptamine-free clones were also free of β-carbolines, but contained gramine and hordenine (D. Woods, unpublished data). Simons and Marten (1971) reported a highly negative correlation between palatability and total alkaloid concentration. Marten et al. (1976, in press) found poor weight gains and a high incidence of diarrhea in sheep and cattle grazing high alkaloid pastures. Animal performance was poorest on tryptamine-carboline containing pastures. Thus, production of a reed canarygrass variety free of tryptamines and carbolines and low in gramine and hordenine appears worthwhile and feasible.

The primary objective of the present study was to find tryptamine-carboline-free, low-gramine genotypes of reed canarygrass which could be utilized in breeding programs. This study also describes an

improved method for gramine determination.

MATERIALS AND METHODS

Plant Material

Seed of reed canarygrass (Phalaris arundinacea L.) introductions was obtained from the USDA North-Eastern Regional Plant Introduction Station, Geneva, New York and the Western Regional Plant Introduction Station, Pullman, Washington.

Five clones of the cultivar Grove (formerly Ottawa syn-F) and two of Ottawa syn-C were established in a replicated space planted (91 cm centers) nursery in 1970. Seedlings of the cultivar Rise and of 16 introductions were started in greenhouse flats in the spring of 1971. Fifty plants of each line were transplanted in a non-randomized field nursery. This procedure was repeated in 1973 with 34 additional introductions.

Sampling

The nursery established in 1971 was sampled during 1971 for determination of the number of tryptamine-carboline containing (TC+) plants. Subsequent sampling of tryptamine-carboline free (TC-) plants (15 from each strain in 1974, 10 in 1975) was conducted at a time when the plants were fully headed. Approximately 3-week old regrowth material was taken in late August of 1974 and 1975 as samples from the nursery established in 1973. Similarly, 3-week regrowth material was taken in 1975 from the variety Rise in the 1971 nursery. The clones of Grove and Ottawa syn-C were sampled only in 1975, at which time both fully headed and regrowth material was taken.

Sample size was approximately 5-10 gm fresh weight. Samples

consisted of leaf blades in the regrowth samples, and of a sub-sample from the chopped complete forage in the case of samples from fully headed plants.

Analyses Performed

Preliminary screening of approximately 50 plants from each strain was conducted to determine the proportion of TC- plants. Where possible, 10 TC- plants were selected from each strain for quantitative determinations of gramine levels in 1974 and 1975. Eleven to 15 TC- plants from each strain were quantitatively analyzed in 1974 from the 1971 nursery. The 7 clones from Grove and Ottawa syn-C had been selected as TC- from a nursery of progenies of the parental clones of these synthetics which had been planted in 1969 to determine the proportion of TC- plants. The 4 replicates of these 7 clones were analyzed separately and the values were averaged.

Qualitative Detection of Tryptamines

The presence or absence of tryptamines and carbolines in the samples was determined by the method described by Woods and Clark (1971a). Approximately 3 gm of frozen sample were used for this procedure.

Quantitative Extraction Procedure

The extraction procedure used was a modification of that described by Woods and Clark (1971a). Approximately 5-10 gm of frozen grass was chopped into pieces 1-3 cm in length and extracted overnight (16 hours) in 100 ml of a mixture of chloroform, methanol and concentrated ammonium hydroxide (26:33:1). Duplicated samples of 10 ml were removed and purified in large test tubes (20 x 175 mm) by the following procedure:

1. Sulfuric acid, 10 ml of 2N, was added and the contents of the tubes were stirred. The pigmented chloroform layer was removed to waste.
2. Chloroform, 10 ml, was added and, after settling, removed to waste.
3. Concentrated ammonium hydroxide, 3 ml, was added followed by 10 ml of chloroform and the contents were stirred. The aqueous layer was discarded.
4. Water, 10 ml, was added, the contents were stirred and the aqueous layer discarded, leaving 10 ml of chloroform containing the extracted bases.

Colorimetric Assay for Gramine

1. To the 10 ml of chloroform from Step 4 above, 5 ml of 10N HCL were added and after stirring, the chloroform removed to waste.
2. Xanthydrol reagent (suspension of 5 mgm xanthydrol/ml of 95% ethanol), 1 ml, and 1 ml of 95% ethanol were added. The tubes were shaken and let stand for 3 hours.
3. Sodium metabisulfite (12.5% aqueous), 2 ml, was added and the tubes cooled in ice for 1 hour.
4. The solution was filtered through Whatman #1 filter paper.
5. The optical density (OD) was measured at 500 nm, using 12 mm internal diameter cuvettes in a Spectronic 20 spectrophotometer (Bausch and Lomb, New York).

This is a modification of the gramine assay described by Woods and Clark (1971a). It gives a linear relationship between gramine

content and OD.

Calculation of Results

The observed sample OD was compared with a calibration line to determine the amount of gramine. The calibration line was a least squares fit to reference samples of variable gramine concentrations, with 200 ug of gramine per sample having an OD of 1.0.

The extraction efficiency of gramine in the quantitative extraction procedure was determined to be 87%. This efficiency was taken into account in the following formula for determining gramine concentration:

$$\text{ug gramine/gm DM} = \text{OD} \times 200 \times \frac{1}{0.87} \times \frac{1}{\text{sample dry wt.}}$$

Statistical Analyses

Correlation coefficients were determined from gramine data between years, a separate coefficient being calculated for samples of fully headed and regrowth forage. A correlation coefficient was also calculated between full heading and regrowth data for the 1975 samples from the clones selected from the Ottawa synthetics. Introduction and variety means and ranges were calculated for each year.

RESULTS

Presence of Tryptamines

Of the 2,471 plants screened in this study, 1,215 were found to be TC+ and 1,256 were TC- (Tables 1 and 2). There were large differences in the numbers of TC+ and TC- plants among introductions. In three introductions, P.I. 172443 from Turkey, P.I. 235547 from Sweden and P.I. 253317 from Yugoslavia, all 50 plants screened were TC+.

All 50 plants of P.I. G14795 from New York and P.I. 284179 from France were TC-. In the variety Rise, 38 out of 45 plants were TC-, while in Grove, slightly more than half of the sampled plants were TC-.

Gramine Levels Between Years and Sampling Times

Mean gramine levels of the introductions and cultivars sampled at full heading were considerably lower than those sampled at regrowth (Tables 1 and 2). Mean levels of gramine in samples of regrowth were slightly higher in 1975 than 1974, while in the samples of fully headed plants, 1974 levels were more than twice those of 1975. The mean gramine concentration over two years for samples of fully headed plants was 810 $\pm$ 90 ug/gm DM (0.081% $\pm$ 0.009% of dry weight) and 2150 $\pm$ 130 ug/gm DM (0.215% $\pm$ 0.013% of dry weight) for regrowth samples.

Simple correlation coefficients calculated between the 1974 and 1975 data were highly significant (p=0.01). The coefficient for samples from fully headed plants was 0.82 (n=93) and for regrowth samples it was 0.72 (n=254). The simple correlation coefficient between the full heading and regrowth stages in 1975 was 0.80 (significant p=0.05). This value was determined from data on the Grove and Ottawa syn-C clones. These were the only plants sampled at both stages.

Strain Variability for Gramine Concentration

There were large differences in mean gramine concentration among the reed canarygrass strains included in this study (Tables 1 and 2). Among the strains sampled at full heading, introductions P.I. 234694

from Denmark and P.I. 251841 from Austria were the lowest in gramine. Introduction P.I. 227670 from Iran had a mean gramine concentration of 2560 ug/gm DM which was above the mean of regrowth lines. Rise was lower in mean gramine concentration than the mean of strains sampled at full heading, while Grove was higher in gramine than all but 2 of the 12 strains sampled in 1975. Only two introductions of this set had mean gramine concentrations in excess of 1000 ug/gm DM (0.1% dry weight).

Among the strains from which regrowth was sampled (Table 2), six introductions had mean gramine concentrations less than 1000 ug/gm DM. Rise was among the highest in gramine concentration, while Grove was among the lowest. This was opposite to samples from fully headed plants, where gramine concentration of Grove was higher than that of Rise.

Interplant Variability for Gramine

Among the individual plants analyzed, gramine levels ranged from 0 to 10940 ug/gm DM (1.094% of dry weight) in 1974 and 30 to 11510 ug/gm DM in 1975. Within most strains large interplant variation in gramine concentration was found. Even in P.I. 227670 from Iran which contains a high mean gramine level, plants very low in gramine were found. In fact, in 1974 this particular introduction contained a plant with no detectable gramine as well as the plant with the highest gramine concentration. P.I. G14665 from New York also contained an apparently gramine-free plant in 1974. In 1975, however, these two plants were shown to contain low levels of gramine.

DISCUSSION

There appear to be a large number of diverse reed canarygrass lines

from which to select plants free of tryptamines and carbolines. Over half of the almost 2,500 plants screened were TC- and only three introductions were entirely TC+. Since it has been shown that the presence of tryptamines is controlled by a single dominant gene (Woods and Clark, 1971a) tryptamines can easily be eliminated from a breeding population by removing TC+ plants. Greenhouse seedlings can be screened and TC+ plants eliminated before transfer to a field nursery.

Both Rise and Grove were found to be tryptamine and carboline containing cultivars, but Rise was found to contain a lower percentage of TC+ plants than Grove. This is of interest, since palatability was one of the criteria used in the selection of clones for Rise (R.R. Kalton, Land O'Lakes Research, personal communication). Hovin and Marten (1975) screened six cultivars and found Vantage to be the only cultivar free of tryptamines and carbolines. In contrast to the present study, these workers found Rise and Grove to contain similar percentages of TC- plants. They also found Grove to be significantly lower in total alkaloid concentration (averaged over regrowth and first growth samples) than Rise or Vantage.

A wide interplant variation in gramine concentration was found in the sampled material. Many lines, including Rise and Grove, contained plants with less than 1000 ug/gm (0.1% dry weight) gramine. Gramine data were fairly highly correlated between years at both the fully headed and regrowth stages. A high correlation also was found between first growth and regrowth data, although this was calculated from a small number of plants. Thus, it appears that broad sense

heritability for gramine concentration in reed canarygrass is quite high. Barker and Hovin (1974) reported generally large narrow sense heritability estimates (h^2 = 0.36 to 1.23) for total alkaloid concentration in reed canarygrass. They found narrow sense estimates to be almost as high as corresponding broad sense estimates indicating high additive genetic variance. Oram (1970) reported a high narrow sense heritability estimate (h^2 = 0.92) for alkaloid concentration in a related species, Phalaris aquatica L. Thus, the possibility of producing strains low in gramine on the basis of screening space planted nurseries in a single year appears promising.

The generally higher gramine concentration of regrowth material was partly due to sampling largely leaf blades, while at full heading, many stems and sheaths were included. Hagman et al. (1975) found leaf blades to contain more than twice the alkaloid concentration of stems and sheaths. Furthermore, they, as well as Woods and Clark (1971a), found regrowth material to be substantially higher in alkaloid concentration than original growth during a specific season. Although high year-to-year correlations were found for both regrowth and first growth samplings in the present study, we would recommend sampling of leafy regrowth because a more uniform sample can be obtained. Our recommendation agrees with that of Hagman et al. (1975), who suggested sampling the leafy upper third of the total herbage to gain greatest uniformity of alkaloid concentration.

Our own experience (unpublished) and that of others (Audette et al., 1970; Williams et al., 1971), has indicated that hordenine is also a

major alkaloid of reed canarygrass. No research has been done, however, to examine the effect of this alkaloid on animal performance. We have recently developed a method to rapidly screen plants for hordenine concentration and initial results indicate a wide interplant variation for this alkaloid.

Recent work by Marten et al. (1976, in press) has clearly demonstrated the relationship of alkaloids to the quality of reed canarygrass. They compared four pasture types, two of which were TC+ (one high in tryptamines, and one low) and two which were TC- (one high in gramine, and one low). The total alkaloid concentration was highly negatively associated with average daily gains (ADG) by grazing lambs and steers ($r = -0.90$ or greater). The total alkaloid concentration was also highly negatively correlated with voluntary intake by lambs that were not offered a choice of genotypes. This latter relationship was not found with steers, indicating lambs are more sensitive to alkaloid differences. Diarrhea in lambs and steers was more prevalent on the TC+ pastures, and the TC+ genotypes were more often associated with poor animal performance than the TC- genotypes.

Thus, production of cultivars free of tryptamines and carbolines and with lower levels of gramine and perhaps hordenine should result in improved palatability and animal performance. The present study has identified diverse genotypes free of tryptamines and carbolines and showing a wide range of gramine levels from which interested breeders may select source materials. Even in our present varieties, improvements could be made by selection of tryptamine-carboline free

genotypes lower in gramine concentrations.

ACKNOWLEDGMENTS

Financial support from the Canada Department of Agriculture and the National Research Council of Canada is gratefully acknowledged.

TABLE 1. Alkaloid analysis data for reed canarygrass introductions and varieties sampled at full heading.

P.I. Number	Geographic Origin	No. of Plants TC+	No. of Plants TC−	Gramine Concentration (ugm/gm DM) 1974 No. Plants	1974 Mean	1974 Range	1975 No. Plants	1975 Mean	1975 Range	1974 1975 Mean
235546	Sweden§	14	35	13	800	370– 1780	10	280	100– 630	570
234694	Denmark§	30	20	15	300	120– 600	10	130	40– 210	230
251841	Austria§	12	38	11	150	50– 220	10	330	110– 990	240
209979	Siberia§	30	20	12	390	140– 1120	10	230	40– 480	320
227670	Iran§	4	46	15	3100	0–10940	10	1740	610–3020	2560
G14665	New York§	16	33	15	1370	0– 3960	10	680	300–1250	490
G18871	New York§	26	19	12	390	40– 1050	10	200	50– 390	300
G14644	New York§	26	23	13	1900	100– 3730	9	1010	70–1730	1540
241064	Oregon∞	32	18	14	860	440– 1820	10	200	30– 540	590
Rise	Indiana$^{\Psi}$	7	38	14	820	160– 2240	10	150	60– 370	540
Grove	Ottawa$^{\Phi}$	82*	94*	—	—	— —	5	690	540–1000	—
Syn C	Ottawa$^{\Phi}$	103*	74*	—	—	— —	2	450	380– 510	—
172443	Turkey∞	50	0†							
269728	Illinois§	39	10†							
236525	Portugal§	12	36†							
316330	Portugal∞	15	32†							
284179	France§	0	50†							
235482	Switzerland§	31	19†							
253315	Yugoslavia§	25	25†							
Total Means or Ranges		554	630	134	1050	0–10940	106	490	30–3020	810

* Determined from a larger nursery in 1971.

† No further analyses due to failure to survive.

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TABLE 2. Alkaloid analysis data for reed canarygrass introductions and varieties sampled at 3 week regrowth.

P.I. Number	Geographic Origin	No. of Plants TC+	No. of Plants TC−	Gramine Concentration (ugm/gm DM) 1974 No. Plants	1974 Mean	1974 Range	1975 No. Plants	1975 Mean	1975 Range	1974 1975 Mean
G14667	New York§	22	28	10	3570	2230–5580	10	4590	2530–8000	4080
G14666	New York§	25	25	10	4140	2100–6250	10	2700	1220–5660	3420
G14795	New York§	0	50	10	2920	1240–5350	9	2170	1240–3440	2550
G18872	New York§	10	39	10	810	350–1670	10	590	190–1080	700
G19665	New York§	1	49	10	1520	660–2210	9	1540	660–2400	1530
G19176	Canada§	12	38	10	910	70–2720	10	930	130–1940	920
235547	Sweden§	50	0	—	——	—— ——	—	——	—— ——	——
234790	Sweden§	18	31	9	2280	890–3710	9	1050	470–2360	1670
234695	Denmark§	47	3	2	1740	1000–2480	2	1020	550–1480	1380
234696	Denmark§	49	1	—	——	—— ——	—	——	—— ——	——
234697	Denmark§	47	3	3	2550	2400–2640	3	1710	1310–2250	2130
235551	Denmark§	48	2	2	710	600– 820	2	510	210– 800	610
235023	Germany§	42	8	8	2720	1460–4710	8	3330	1170–6630	3030
234780	Germany§	30	20	9	1740	800–3190	8	2000	530–4600	1870
237724	Germany§	2	45	10	2010	490–5760	10	3550	1410–11510	2780
225116	Germany§	27	23	10	1780	880–3840	10	1400	530–2280	1590
235483	Switzerland§	11	39	10	1320	440–2280	10	900	270–1510	1110
235484	Switzerland§	36	14	10	2270	1410–5100	10	1300	550–2050	1790
235485	Switzerland§	32	17	10	2240	1110–4190	10	3900	2000–5250	3070
251841	Austria§	5	45	11	1740	530–2930	10	1650	170–2530	1700
251842	Austria§	15	35	10	1110	460–2260	10	1230	530–2780	1170
253316	Yugoslavia§	15	35	10	750	220–2120	10	750	300–1560	750
253317	Yugoslavia§	50	0	—	——	—— ——	—	——	—— ——	——
251426	Yugoslavia§	14	36	9	2610	740–4660	9	2990	2200–4550	2800
251531	Yugoslavia§	20	30	10	750	280–1370	10	640	230–1090	700
255887	Yugoslavia§	19	30	10	1280	690–2130	10	1130	760–1880	1210
253315	Yugoslavia∞	33	17	10	1600	800–2400	10	2800	1320–4340	2200

Cont'd.

TABLE 2. Continued.

P.I. Number	Geographic Origin	No. of Plants TC+	No. of Plants TC-	Gramine Concentration (ugm/gm DM) 1974 No. Plants	1974 Mean	1974 Range	1975 No. Plants	1975 Mean	1975 Range	1974 1975 Mean
315487	U.S.S.R.[§]	41	9	8	870	40-3030	6	2020	700-5130	1450
319825	Norway[§]	41	8	5	650	300-1140	5	1330	810-1620	990
241064	Oregon[§]	39	11	5	1090	360-2320	5	2050	1600-2510	1580
337718	U.S.S.R.[§]	2	48	9	3470	2300-6940	9	4160	2210-5900	3820
314102	U.S.S.R.[§]	9	41	11	2830	1960-4770	10	4510	1720-6370	3670
315486	U.S.S.R.[§]	33	15	10	1510	390-2510	10	1920	710-3500	1720
Rise	Indiana[Ψ]	7	38	—	——	—— ——	8	2880	980-7140	——
Grove	Ottawa [Φ]	82*	94*	—	——	—— ——	5	1090	640-1750	——
Syn C	Ottawa [Φ]	103*	74*	—	——	—— ——	2	590	450- 720	——
Total or Mean		853	832	261	2090	40-6940	266	2200	170-11510	2150

* Determined from a larger nursery in 1971.

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M A N U S C R I P T II

THE EFFECT OF SELECTED REED CANARYGRASS ALKALOIDS ON IN VITRO DIGESTIBILITY

THE EFFECT OF SELECTED REED CANARYGRASS ALKALOIDS ON IN VITRO DIGESTIBILITY

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ABSTRACT

A range of concentrations of the reed canarygrass alkaloids, gramine, hordenine, 5-methoxy-dimethyl tryptamine and N-methyl tryptamine were added to *in vitro* rumen fluid fermentations. Powdered cellulose and ground reed canarygrass were used as substrates and fermentation periods were 24 and 48 hours. No consistent relationship was found between the concentration of any of the above alkaloids and *in vitro* digestion values for either of the substrates or fermentation periods.

In other studies, 24 and 48 hour *in vitro* digestions were carried out on reed canarygrass clones harvested at early heading and 6-week regrowth. These clones showed a very wide range of gramine, hordenine and total alkaloid levels. No relationship was found between the concentration and type of alkaloids in the clones and their *in vitro* digestibility. It was concluded that reported poor animal performance with reed canarygrass is not due to alkaloid interference with the rumen microflora.

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INTRODUCTION

Reed canarygrass has considerable potential as a forage crop (Vose, 1959). Erratic seed yields (Vose, 1959) and reports of poor palatability (Rogler, 1944; Marten and Donker, 1968) and poor animal performance (Van Arsdell et al., 1954; Marten and Jordan, 1974) as compared to other cool season grasses have hindered its widespread acceptance. Nine alkaloids (gramine, hordenine, 4 tryptamine derivatives and 3 β-carbolines) have been reported in reed canarygrass (Marten, 1973; Gander et al., 1976). Simons and Marten (1971) reported highly negative correlations between palatability and total alkaloid content. Marten et al. (1976) reported poor intakes and weight gains with lambs and poor weight gains with steers grazing high alkaloid pastures. This study also showed animal performance to be poorest on pastures containing tryptamines and β-carbolines.

Tall fescue (Festuca arundinacea Schreb.) is another alkaloid containing species for which reports of poor animal performance are common (Buckner and Cowan, 1973). Perloline, the major alkaloid of this species, has been shown to inhibit in vitro cellulose digestion and the growth of rumen bacteria, at concentrations sometimes found in the forage (Bush et al., 1970, 1972). This interference with the rumen microflora may explain the poor intakes and performance with this species.

In a review, Marten (1973) reported unpublished data from the University of Minnesota showing no relationship between alkaloid

concentration of the forage and *in vitro* digestibility (IVD). When pure samples of gramine and the dimethylated tryptamines were added to various forages only 5 methoxy-N,N-dimethyl tryptamine (5MeO-DMT) in very high concentrations (3.0% DW) had any effect on IVD. G. W. Arnold (CSIRO, Wembley, Western Australia, personal communication), however, reported a very marked depression of IVD with concentrations of gramine sometimes found in reed canarygrass.

The objectives of the present study were to: 1) Determine the effect on *in vitro* digestibility of added pure samples of hordenine, gramine, 5 methoxy-dimethyl tryptamine (5MeO-DMT), and N-methyl tryptamine (NMT) at concentrations found in reed canarygrass; and 2) Determine the relationship of the alkaloid content and type of reed canary clones to their *in vitro* digestibility.

MATERIALS AND METHODS

For the *in vitro* digestions, rumen fluid was obtained from a fistulated steer. Ten ml of rumen fluid were mixed with 20 ml of a buffer-mineral solution (Baumgardt *et al.*, 1962) in digestion tubes. The substrates used were powdered wood cellulose (Alpha Cell-Nutritional Biochem. Corp.) and ground leaf tissue of reed canarygrass (*Phalaris arundinacea* L.). Substrate quantity in each fermentation tube was 0.5 gm. The reed canarygrass had previously been air dried and ground in a Wiley Mill equipped with a 1 mm screen. This sample was free of tryptamines and β-carbolines, but contained 1380 ugm/gm DM gramine (0.138%) and 1740 ugm/gm hordenine (0.174%).

Pure samples of gramine (J.T. Baker Chem. Co.), NMT (Aldrich Chem. Co.), 5MeO-DMT and hordenine (Sigma Chem. Co.) were dissolved

in 5% (v/v) acetic acid. One ml of alkaloid solution and/or 5% acetic acid were added to each digestion tube prior to the beginning of the fermentation period. The addition of the acid had no effect on pH as the initial and final pH's of the fermentation media were between 6.5 and 7.0. Four different concentrations of each alkaloid were used, in accordance with concentrations normally reported for reed canarygrass. Fermentation times of 24 and 48 hours were used for all treatments. All treatments were done in triplicate and the closest two values averaged. Three controls (i.e. no addition of alkaloid) were included in each fermentation period and the closest two values averaged. Percent organic matter digested in vitro (%IVDOM) was determined on the reed canarygrass substrate. This was not a complete organic matter digestion as no acid-pepsin digestion was included in the procedure. In the case of the cellulose substrate, values were expressed as % in vitro digestible cellulose (%IVDC).

A space planted nursery (91 cm centers) of reed canarygrass introductions was established in 1973. In 1974, plants of this nursery were screened for gramine concentrations and the presence of tryptamines and β-carbolines. Eight tryptamine-carboline free plants with variable levels of gramine and two tryptamine-carboline containing plants were sampled in 1974 at: 1) early heading, and 2) 6-week regrowth (plants approximately 30 cm in height). Determinations of gramine and hordenine levels (Coulman et al., 1976, in press) were made on fresh frozen tissue of these samples. A portion of each sample was air dried and ground to pass through a 1 mm screen. Determinations of acid-detergent fiber % (ADF%) and % IVDOM

were made with this dried material. For the *in vitro* digestions 24 and 48 hour fermentation periods were used with no additional acid-pepsin digestion.

One-way analyses of variance were done on duplicate values for IVDOM for each alkaloid at each digestion period and least significant differences (LSD's) were determined. Simple correlation coefficients were calculated between IVDOM values and alkaloid and ADF levels, a separate coefficient being calculated for first growth and regrowth samples. Correlation coefficients were also calculated for each variable between the two harvests. Clonal means and ranges for each variable were determined in each harvest.

RESULTS

Effect of Addition of Alkaloids to Fermentation Media

Using the cellulose substrate, significant differences ($p=0.05$) in IVDC were found between concentrations of each alkaloid for the 48 hour digestion (Table 3). For the 24 hour digestion, no significant differences were found between concentrations of gramine but there were significant differences between concentrations of the other three alkaloids. There was, however, no consistent relationship between IVDC and the concentration of any of the alkaloids tested.

Using the reed canarygrass substrate, significant differences ($p=0.05$) in IVDOM were found between concentrations of gramine for both the 24 and 48 hour digestions (Table 4). No significant differences in IVDOM were found among concentrations of the other three alkaloids. For the 24 hour digestion, the highest alkaloid

concentration consistently produced the lowest IVDOM value. In no case, however, was this value significantly lower than the IVDOM of the control. For the 48 hour digestion, there was, again, no consistent relationship between IVDOM and alkaloid concentration.

Analysis of Selected Genotypes

Wide ranges of gramine, hordenine and total alkaloid (gramine + hordenine) levels were found among the samples genotypes (Tables 5 and 6). Plant 18-34 had the highest total alkaloid level, 6820 ugm/gm DM (0.682%) at regrowth, while 24-12 had the lowest, 630 ugm/gm DM (0.063%). The mean gramine level of regrowth sampled plants was more than twice that of first growth, while hordenine levels were similar at both samplings. The mean ADF levels were higher and IVDOM levels lower in the regrowth samples. Greater ranges of IVDOM and ADF were found in first growth. Plant 6-24 produced very little regrowth material and thus, only a first growth sample was taken.

Correlations Among Variables

Correlation coefficients between the 24 hour IVDOM and ADF, gramine, hordenine and total alkaloids were non-significant (Table 7). Similar results were found for the correlation between the 48 hour IVDOM and these variables. IVDOM values for the 24 and 48 hour fermentation periods were not significantly correlated.

Between the two harvests (Table 8), correlation coefficients were highly significant ($p=0.01$) for ADF, gramine and total alkaloid levels of the clones and significant ($p=0.05$) for hordenine levels. Twenty-four hour IVDOM values were not significantly correlated

between harvests, while 48 hour values were significantly negatively correlated ($p=0.05$).

DISCUSSION

The addition of the reed canarygrass alkaloids, hordenine, gramine, 5MeO-DMT and NMT to *in vitro* digestion media appears to have little effect on the activity of the rumen microflora. This is in contrast to results for perloline, the major alkaloid of tall fescue (Bush *et al.*, 1970, 1972). Concentrations of perloline up to 7×10^{-4}M were used and *in vitro* cellulose digestion was almost totally inhibited with a concentration of 2×10^{-4}. These concentrations were said to be realistic with those often found in tall fescue (up to 6700 ugm/gm DM). Range of molarities of the alkaloids used in the present study were: hordenine, $1.2 - 4.8 \times 10^{-4}$; gramine, $2.3 - 9.3 \times 10^{-4}$; 5MeO-DMT, $1.9 - 7.4 \times 10^{-5}$; NMT, $2.3 - 9.3 \times 10^{-5}$. Thus, at similar concentrations perloline is much more toxic to the rumen microflora than the alkaloids used in the present study.

Bush *et al.* (1970) found the inhibitory effects of perloline on IVDC were greater with a cellulose substrate than with tall fescue substrate. It was suggested that there may be some factor present in the tall fescue to reduce the inhibitory effects of perloline. In the present study, the digestibility of the cellulose was, of course, lower than the reed canarygrass, but no difference was found in the relationship of these substrates to alkaloid concentration.

G. W. Arnold (CSIRO, Wembley, Western Australia, personal communication) reported a marked inhibition of IVD of orchardgrass (*Dactylis glomerata* L.) with addition of gramine levels of 10000 ugm/gm

DM (1.0%). In the present study, the 24 hour IVDOM of reed canarygrass appeared to be somewhat depressed with this level of gramine, but this depression was not seen in the 48 hour IVDOM. However, gramine levels as high as 10000 ugm/gm DM occur very rarely in reed canary genotypes (Coulman et al., 1976, in press). A study of varieties (Hovin and Marten, 1976) showed that total alkaloid contents, averaged over two cuts, ranged from 1180 - 1700 ugm/gm DM. Marten (1973) found no inhibitory effect on IVD of added gramine up to 30000 ugm/gm DM, but 5MeO-DMT at this concentration did significantly depress IVD. The chances of finding such a high concentration of 5MeO-DMT are unlikely since tryptamine levels in both reed canarygrass and hardinggrass (Phalaris aquatica L.) have usually been reported to be less than 1000 ugm/gm DM (Barnes et al., 1971; Woods, 1971; Moore, Williams and Chia, 1966; Oram, 1970).

No consistent relationship was found between alkaloid content and IVDOM of selected reed canarygrass clones. Correlation coefficients calculated from such small numbers of observations (i.e. 7-10) must, of course, be interpreted with caution. However, the fact that such a wide range in alkaloid content (630 - 6820 ugm/gm DM at regrowth) produced no consistent differences in IVDOM, points out the minor importance of this factor in determining digestibility. These results agree with findings of Marten (1973) for a number of reed canary clones. Alkaloid type was also not an important factor as the two clones containing tryptamines in the present study were not consistently lower in IVDOM than those free of tryptamines.

Alkaloid levels were highly correlated between harvests. This

is in accordance with reports of low genotype x environment interactions for total alkaloid content (Barker and Hovin, 1974; Marten et al., 1973). IVDOM, however, was not consistent between harvests and the 48 hour IVDOM values were significantly negatively correlated between cuts.

From the results presented in the present study and those previously published (Marten, 1973), it appears that the poor animal performance often reported with reed canarygrass is not due to alkaloidal interference with the rumen microflora. A more likely cause of poor weight gains is the physiological disturbance, as evidenced by the occurrence of diarrhea, that has been reported in animals grazing reed canarygrass (Marten et al., 1976; Woods and Clark, 1974). A higher incidence of diarrhea was found on high alkaloid pastures and especially on those pastures containing tryptamines and β-carbolines. The dimethyl tryptamines are thought to interfere with the normal functions of serotonin (Gallagher et al., 1964), while β-carbolines inhibit monoamine oxidase (Ho et al., 1968), the enzyme responsible for controlling levels of serotonin and other amines in the body. A build up of serotonin could lead to hyper-excitability and increased respiration, thus burning up food reserves. Woods and Clark (1974) found that under heat stress (24-27°C), sheep respiration rates were higher on a tryptamine-carboline containing pasture than on a pasture free of these compounds.

ACKNOWLEDGMENTS

Financial support from the Canada Department of Agriculture and the National Research Council of Canada is gratefully acknowledged. The authors would also like to thank the Manitoba Department of Agriculture for acid-detergent fiber analyses.

TABLE 3. Twenty-four and 48 hour percent *in vitro* digestibility of a cellulose substrate under varying concentrations of four alkaloids.

Alkaloid	Concentration (ug/gm dry cellulose)	24 Hour Digestibility	48 Hour Digestibility
Hordenine	00	35.1	52.9
	1250	37.7	46.9
	2500	37.8	51.2
	3750	34.6	47.5
	5000	36.9	49.9
LSD(p=0.05)		2.3	2.0
Gramine	00	35.1	52.9
	2500	39.4	46.6
	5000	36.7	49.8
	7500	38.9	45.5
	10000	37.1	50.5
LSD(p=0.05)		N.S.	3.6
5MeO-DMT	00	35.1	52.9
	250	38.9	46.8
	500	39.2	49.5
	750	38.6	44.5
	1000	35.1	53.8
LSD(p=0.05)		3.3	4.0
NMT	00	35.1	52.9
	250	33.8	50.4
	500	32.6	53.2
	750	36.4	47.5
	1000	36.7	48.9
LSD(p=0.05)		1.7	2.6

TABLE 4. Twenty-four and 48 hour percent *in vitro* digestible organic MATTER (% IVDOM) of a reed canarygrass substrate* under variable concentrations of four alkaloids.

Alkaloid	Concentration (ug/gm D.M.)	24 Hour Digestibility	48 Hour Digestibility
Hordenine	1800	51.1	58.8
	2500	52.2	61.2
	3750	53.2	60.3
	5000	49.7	60.8
LSD(p=0.05)		N.S.	N.S.
Gramine	1400	51.1	58.8
	2500	55.0	60.1
	5000	54.0	60.3
	7500	49.6	58.9
	10000	47.8	60.2
LSD(p=0.05)		4.6	2.4
5MeO-DMT	00	51.1	58.8
	250	51.7	60.6
	500	53.9	58.7
	750	50.2	59.5
	1000	46.8	61.2
LSD(p=0.05)		N.S.	N.S.
NMT	00	51.1	58.8
	250	52.8	58.8
	500	51.0	60.0
	750	48.4	58.6
	1000	49.0	59.9
LSD(p=0.05)		N.S.	N.S.

* Contains 1740 ug/gm D.M. hordenine and 1380 ug/gm D.M. gramine.

TABLE 5. Alkaloid contents, ADF %, and % IVDOM (24 and 48 hour digestions) of reed canarygrass clones cut at early heading.

Clone	Gramine (ugm/gm D.M.)	Hordenine (ugm/gm D.M.)	Total Alkaloid (Gram. + Hord.)	ADF %	% IVDOM 24 Hour Digestion	% IVDOM 48 Hour Digestion
18-34	2410	1850	4260	27.5	39.4	54.2
22-22	190	2220	2410	27.0	38.4	52.6
2-39	860	890	1750	28.3	46.3	53.6
6-24	120	1360	1480	30.6	41.3	54.8
22-15	230	1060	1290	30.6	41.2	59.7
30-11	150	810	960	35.7	41.8	54.2
30-23	140	820	960	33.5	41.0	60.1
24-12	30	610	640	33.2	44.1	62.8
6-27	— *	640	—	29.4	41.8	55.6
6-29	— *	560	—	28.0	38.7	51.7
Mean	520	1080	1720	30.4	41.4	55.9
Range	30 - 2410	560 - 2220	640 - 4260	27.0-35.7	38.4-46.3	51.7-62.8

* No gramine analyses due to presence of tryptamines.

TABLE 6. Alkaloid contents, ADF %, and % IVDOM (24 and 48 hour digestions) of reed canarygrass clones cut at six-week regrowth.

Clone	Gramine (ugm/gm D.M.)	Hordenine (ugm/gm D.M.)	Total Alkaloid (Gram. + Hord.)	ADF %	% IVDOM 24 Hour Digestion	% IVDOM 48 Hour Digestion
18-34	3760	3060	6820	33.7	36.3	51.4
2-39	1860	1030	2890	36.1	39.4	52.4
22-22	570	1670	2240	37.9	36.6	48.4
30-11	530	1190	1720	39.2	36.1	50.5
22-15	510	940	1450	37.2	37.3	48.9
30-23	333	660	990	38.1	36.8	48.1
24-12	80	550	630	38.9	37.0	48.1
6-27	— *	380	—	36.0	36.3	47.5
6-29	— *	550	—	34.4	37.9	52.3
Mean	1090	1120	2390	36.8	38.0	49.7
Range	80 - 3760	380 - 3060	630 - 6820	33.7-39.2	36.1-39.4	47.5-52.4

* No gramine analyses due to presence of tryptamines.

TABLE 7. Correlations between IVDOM and alkaloids or ADF for two harvests of reed canarygrass.

Variable	1st Cut		Regrowth	
	r	n	r	n
IVDOM (24 Hour) vs:				
Gramine Content	-0.16	8	0.07	7
Hordenine Content	-0.49	10	-0.26	9
Total Alkaloid	-0.48	8	-0.09	7
ADF	0.24	10	-0.24	9
IVDOM (48 Hour) vs:				
Gramine Content	-0.37	8	0.74	7
Hordenine Content	-0.36	10	0.33	9
Total Alkaloid	-0.56	8	0.65	7
ADF	0.57	10	-0.56	9
IVDOM : 24 vs 48 hour	0.36	10	0.56	9

TABLE 8. Correlations of variables between two harvests of reed canarygrass.

Variable	r	n
Gramine	0.99 **	7
Hordenine	0.67 *	9
Total Alkaloid	0.96 **	7
ADF	0.75 **	9
IVDOM (24 hour)	-0.31	9
IVDOM (48 hour)	-0.62 *	9

*, ** significant p=0.05 and 0.01 respectively

M A N U S C R I P T III

DISTRIBUTION WITHIN THE PLANT, VARIATION WITH MATURITY, AND HERITABILITY OF GRAMINE AND HORDENINE IN REED CANARYGRASS

DISTRIBUTION WITHIN THE PLANT, VARIATION WITH MATURITY, AND HERITABILITY OF GRAMINE AND HORDENINE IN REED CANARYGRASS

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ABSTRACT

Clones of reed canarygrass (Phalaris arundinacea L.) which were free of tryptamines and carbolines, were divided into blades, sheaths and stems and analyzed for gramine and hordenine. Gramine concentrations in leaf blades were over 5 times as high as those in sheaths or stems. Hordenine was highest in the leaf sheaths, being over twice as high as in blades and over 4 times as high as in stems. These alkaloids were also determined in leaf blades of reed canarygrass of varying maturity. Both gramine and hordenine increased slightly from a vegetative stage to early heading in samples of first growth. The concentrations of both alkaloids increased considerably in new regrowth, but in more mature regrowth, had declined to levels found in first growth. Under greenhouse conditions, narrow sense heritability estimates were 0.72 for gramine and 0.53 for hordenine. Hordenine was shown to be a major alkaloid of reed canarygrass as concentrations were higher than those of gramine in most of the genotypes included in this study. A colorimetric

assay for the estimation of hordenine is described.

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INTRODUCTION

Nine alkaloids (gramine, hordenine, 4 tryptamine derivatives and 3 β-carbolines) have been identified in reed canarygrass (Phalaris arundinacea L.) (Marten, 1973; Gander et al., 1976). Concentrations of these alkaloids are negatively correlated with palatability (Simons and Marten, 1971). Poor weight gains and a high incidence of diarrhea have been reported for sheep and cattle grazing high alkaloid pastures (Marten et al., 1976).

In a study of alkaloid distribution within the plant, Hagman et al. (1974) found concentrations in leaf blades to be more than twice as high as those in stems and sheaths. These workers also found alkaloid concentration to increase slightly with maturity in leaf blades of first growth forage, but decline with maturity in total herbage in both first growth and regrowth. In another study (Barker and Hovin, 1974), total alkaloid concentration in reed canarygrass was shown to be a highly heritable character. The above studies utilized the method of Simons and Marten (1971) for the determination of total alkaloid content. D. Woods (unpublished) has found that this method extracts and estimates the total indole alkaloid concentrations, but very little of the hordenine.

Woods and Clark (1971a) found the presence of tryptamines in reed canarygrass to be controlled by a single dominant gene. The program at the University of Manitoba has been involved in screening tryptamine-free genotypes for concentrations of gramine and hordenine.

Woods and Clark (1971a) found that gramine (on a fresh weight basis) in total herbage increased slightly with maturity in free growth, but increased dramatically throughout the season under continual clipping. Other than the above study, no information exists on the distribution within the plant, seasonal variation and heritability of gramine and hordenine individually.

The objectives of this study were to: 1) Determine the concentrations of gramine and hordenine in plant parts of reed canarygrass; 2) Determine the variation with plant maturity of these alkaloids in leaf blades of first growth and regrowth forage; 3) Determine narrow sense heritability estimates for gramine and hordenine concentrations. A colorimetric assay for the determination of hordenine concentrations was developed.

MATERIALS AND METHODS

Alkaloid Concentration in Plant Parts

Seven tryptamine-carboline free (TC-) genotypes were selected for low gramine concentrations from a nursery of reed canarygrass (Phalaris arundinacea L.) introductions established in 1973. The plants were moved to an isolated, space planted (91 cm centers), 3-replicate polycross in spring 1975. The entire forage above a 5 cm stubble of each plant of this polycross was harvested in September of 1975. The plants were approximately 0.60 m in height but had not headed. The herbage was divided into 5 portions: a) Upper and lower halves of leaf blades; b) Upper and lower halves of leaf sheaths; and c) Stems. Approximately 5-10 gm (fresh weight) of each part and 10 gm of the complete forage of each plant were chopped into pieces

1-3 cm in length for alkaloid analyses.

Herbage was also harvested in 1975 from 3 TC- genotypes which had headed from the space planted introduction nursery established in 1973. Harvesting was done at a time when the plants were approximately 1 m in height and almost all the seed had shattered from the heads. These plants were divided into blade, sheath, stem and head samples and chopped for alkaloid analyses.

Variation of Alkaloid Concentration with Maturity

Four TC- genotypes from the 1973 introduction nursery were used in this study. These genotypes were selected on the basis of a previous screening which showed them to have a wide range of gramine and hordenine concentrations. Samples of leaf blades of each plant were taken at weekly intervals from May 21st to June 18th, 1975. Different areas of the plant were sampled each time to avoid sampling regrowth material from an area already harvested. After sampling on June 18th, the plants were cut back leaving a 5 cm stubble. Samples of leaf blades from 2, 4 and 5 week regrowth were subsequently collected on July 3rd, 18th and 25th. Five to 10 gm of each sample were chopped into pieces 1-3 cm in length for alkaloid analysis.

Heritability of Gramine and Hordenine Concentration

Genotypes used in the heritability study were selected from a space planted (91 cm) nursery established in 1973 of topcross progenies of 8 TC- plants from Grove and Ottawa syn-C. These 8 clones had been topcrossed to TC- plants selected from seedlings of S-6982, a seed retaining strain supplied by Dr. R. P. Knowles of Saskatoon. Thirty-five clones were selected for seed retention from the topcross

progeny nursery in 1975 and open pollinated (OP) seed was harvested from each clone. Sod pieces were taken from each of the 35 selected clones in September, 1975, divided into 4, and planted in 7.62 cm square pots. The pots were arranged in a randomized complete block design (RCBD) on a greenhouse bench. Twenty seedlings grown from OP seed of each selected clone were planted in 5.08 cm square plastic pots in September, 1975. These pots were arranged in a RCBD with 5 replicates of 4 plants.

All greenhouse plants were trimmed to a height of 5 cm every 2 weeks to induce tillering. A fertilizer solution (4 gm of 20-20-20 in 4.5 l water) was applied after each trimming. Temperature was approximately 20°C and fluorescent illumination was used to obtain a 16 hr daylength.

OP progenies were sampled 8 weeks after planting. Plants had been regrowing for 2 weeks at this time and all were approximately the same size (i.e. approximately 15 cm in height and 5 gm fresh weight). The entire herbage was sampled leaving a stubble of 5 cm. The parents brought in as sod from the field had not grown as well as the progenies at this time. They were repotted in 15.24 cm pots, cut back, fertilized and allowed to regrow for an additional 4 weeks. The entire herbage was then sampled (to a height of 5 cm), being somewhat older growth (4 week regrowth) than the progeny samples (2 week regrowth). Approximately 5 gm of chopped (1-3 cm pieces) forage of each sample were used for alkaloid analysis.

Alkaloid Analysis

Extraction of alkaloids and colorimetric assays for gramine were carried out according to the methods described by Coulman et al. (1976).

The following colorimetric assay was used for the determination of hordenine concentrations. It was not affected by the presence of gramine. This assay was carried out on duplicate extracts from each sample.

1.) The 10 ml of chloroform containing the extracted bases (from the quantitative extraction procedure) were evaporated under vacuum to remove the chloroform (some water remained but did not interfere with later steps).

2.) The residue was dissolved in a solution of 2 ml ethanol and 1 ml water.

3.) Diazo reagent (see below), 10 ml, was added and the mixture let stand for 5 min.

4.) Ethanol, 5 ml was added and after mixing the optical density (OD) was measured immediately at 520 nm, using 12 mm internal diameter cuvettes in a Spectronic 20 spectrophotometer (Bausch and Lomb, New York).

The diazo reagent was prepared by the following procedure and is a modification of that described by Smith (1969).

1.) p-nitro aniline solution (0.15 gm in a solution of 4.5 ml HCl and 95 ml water), 100 ml, was cooled on ice.

2.) Freshly prepared sodium nitrite solution (5% w/v), 0.2 ml, was added and after shaking the mixture was let stand for 1 min.

3.) Sodium carbonate solution (20% w/v), 5 ml, was added and after shaking the mixture was used immediately. Fresh reagent was prepared for each group of 10 samples, since the reactivity of the diazo reagent declined with time.

A linear relationship was found between concentrations of hordenine and OD. The observed sample OD was compared with a calibration line to determine the amount of hordenine. The calibration line was a least squares fit to reference samples of variable hordenine concentrations. A sample of 300 ugm of hordenine had an OD of 1.0.

The extraction efficiency of hordenine in the quantitative extraction procedure was determined to be 66%. This efficiency was taken into account in the following formula for determining hordenine concentration:

$$\text{ugm hordenine/gm DM} = \text{OD} \times 300 \times \frac{1}{0.66} \times \frac{1}{\text{sample dry wt.}}$$

Statistical Analyses

An analysis of variance for a split plot RCBD was carried out on alkaloid data from the 7 clones divided into plant parts. The clones were the main plots and plant parts the sub-plots. Least significant ranges (p=0.05) were calculated between mean values for each plant part. Simple correlation coefficients were calculated between alkaloid concentrations in leaf portions and in the total forage.

Ranges of clonal means and an overall mean were calculated for gramine and hordenine concentrations of parental clones used in the heritability study. Ranges of progeny means and an overall mean for progenies were also determined. Estimates of narrow sense

heritability (h^2) were calculated by correlation of means of parents and progenies (Frey and Horner, 1957).

RESULTS

Alkaloid Concentration of Plant Parts

Leaf blades had significantly higher concentrations of gramine than sheaths and stems (Table 9). No significant differences in gramine concentration were found between upper and lower leaf blades or among upper and lower sheaths and stems. Gramine concentrations in the blade portions were approximately 6–7 times as great as those in the sheath. The total forage was intermediate between blades and sheaths in gramine concentration.

Leaf sheath portions contained a significantly greater hordenine concentration than leaf blades and stems. Leaf blades had significantly higher concentrations of this alkaloid than stems. No significant differences were found between upper and lower blades or between upper and lower sheaths. Hordenine content of leaf sheaths was over twice that found in blades and over 4 times that found in stems. The total forage hordenine was intermediate between that of the blade and sheath. The mean hordenine concentration of the 7 clones, 1220 ugm/gm DM, was considerably higher than the gramine concentration which was 190 ugm/gm DM.

Highly significant correlation coefficients (Table 10) were found between gramine concentrations of the total forage and that of the upper leaf blade (r=0.90) and of the entire blade (r=0.93). Hordenine concentrations of the total forage and upper leaf blade

were significantly correlated (r=0.85) as were concentrations of the total forage and entire blade (r=0.82).

In the 3 sampled clones which had headed out, no gramine was detected in the heads. The leaf blades had a mean gramine concentration of 400 ugm/gm DM, while sheaths and stems contained trace amounts. Mean hordenine concentrations (ugm/gm DM) in these clones were: leaf blade, 430; sheath, 670; stem, 200; and head, 290. Thus, heads contained no gramine but a fairly substantial amount of hordenine.

Alkaloid Variation with Maturity

Concentrations of gramine and hordenine in leaf blades declined slightly after the first sampling on May 21st (Figure 1). Gramine concentrations increased consistently until June 18th while hordenine fluctuated during this period but showed an increase over the first sampling by June 18th. A few heads had begun to appear by June 18th and the forage was cut back. The gramine concentration at this time was 1370 ugm/gm DM and hordenine concentration was 1730 ugm/gm DM.

The samples of 2 week regrowth showed a very marked increase in these alkaloids. Concentrations of 4120 ugm/gm DM gramine and 2500 ugm/gm DM hordenine were found at this sampling. This represented a total alkaloid (gramine + hordenine) concentration of 6620 ugm/gm DM (0.662% DM). Concentrations of gramine and hordenine in 4 week regrowth had declined markedly and were comparable to those found in first growth samples. Hordenine levels in leaf blades of first growth were higher than those of gramine, but

gramine was higher than hordenine in regrowth.

Greenhouse Heritability Study

Means and ranges of hordenine concentrations were considerably greater than those for gramine in both parents and OP progenies included in the greenhouse study (Table 11). Means and ranges for both alkaloids were considerably greater in OP progenies than in parental clones, because the parents were sampled at a more mature stage. Narrow sense heritabilities as determined by parent-progeny correlation (X2) were 0.72 for gramine and 0.53 for hordenine.

DISCUSSION

Gramine was found to be largely confined to the leaf blades of reed canarygrass genotypes sampled in the present study. Hordenine, however, was found to be distributed throughout the plant, with leaf sheaths having the highest concentration. This is an interesting finding since it is the usual situation in alkaloid-containing species to find alkaloids accumulating in the most metabolically active portion of the plant (Robinson, 1974). Our results for gramine agree with those of Hagman et al. (1974) who found total indole alkaloid concentrations of leaf blades of reed canarygrass to be more than twice as high as those in stems and sheaths.

High correlations were found for concentrations of gramine ($r=0.90$ or better) and hordenine ($r=0.80$ or better) between leaf blades and total forage. This suggests that sampling leaf material would be sufficient for detecting relative differences

in these alkaloids among genotypes. This is in agreement with Hagman et al. (1974) who recommended sampling of the leafy upper third of the forage, which they found was highly correlated in total alkaloid concentration to the total forage. If absolute, rather than relative, concentrations of gramine and hordenine in the forage were required, sampling of the entire forage would be necessary. Sampling of only leaf blades would give an inflated estimate for gramine, especially in more mature forage of which stems would make up a greater percentage. In the clones sampled in the present study gramine concentrations in the leaf blades were approximately 50% higher than those found in total herbage. For hordenine, sampling of leaf blades would underestimate concentrations in the entire forage, as illustrated by the present study where blades contained only about 75% of the hordenine concentration of total forage.

Concentrations of hordenine in the 7 clones sampled for the plant distribution study and also in the 35 Grove X S-6982 topcross clones were considerably higher than gramine concentrations. These findings are in agreement with Audette et al. (1970) and Williams et al. (1971) who found hordenine to be a major alkaloid of reed canarygrass. In the present study, however, it should be noted that the 7 clones of the plant distribution study were very low in gramine due to the fact that they had previously been selected for low levels of this alkaloid. There was no evidence of abnormally high concentrations of hordenine in these low gramine plants.

Gramine and hordenine in leaf blades were found to increase

slightly with the maturity of first growth, increase markedly in young regrowth and then decline to levels comparable to those of first growth in more mature regrowth. Hagman et al. (1974) found that total alkaloid concentrations of leaf blades increased slightly in first growth and decreased in regrowth. The marked accumulation of alkaloids in young regrowth found in the present study and also reported by Woods and Clark (1971a) is very significant when reed canarygrass is used for pasture. Under intensive grazing, when forage is constantly being chewed off and regrowing, alkaloid levels would remain very high.

The narrow sense heritabilities reported in the present study for gramine (h^2=0.72) and hordenine (h^2=0.53) are in agreement with those of other workers. Barker and Hovin (1974) reported narrow sense heritability estimates for total alkaloid content ranging from 0.55 to 0.78 for combined results from 2 cuttings. Oram (1970) reported a narrow sense estimate of 0.92 in one environment for tryptamine concentration in Phalaris aquatica L. Whether heritability estimates obtained in the greenhouse can be applied to field data is debatable. However, when one considers the heritability estimates mentioned above, and the high correlations of alkaloid data between field and greenhouse (Hagman et al., 1974; Marten et al., 1974), the estimates obtained in the present study should be comparable to those found in a field study. Thus screening and selection of individual plants for gramine concentration should result in genetic gain. The lower heritability estimate for hordenine may indicate that progeny testing would be necessary for genetic advance in the reduction

of this alkaloid.

ACKNOWLEDGMENTS

Financial support from the Canada Department of Agriculture and the National Research Council of Canada is gratefully acknowledged.

TABLE 9. Mean concentrations (ugm/gm DM) of gramine and hordenine in plant parts of 7 reed canarygrass genotypes.

Alkaloid	Upper Blade	Lower Blade	Upper Sheath	Lower Sheath	Stem	Total Forage
Gramine	300 a	270 a	50 b	40 b	10 b	190
Hordenine	830 a	870 a	1800 b	2040 b	380 c	1220

* Within each row, means followed by the same letter are not significantly different (p=0.05) as determined by Duncan's Multiple Range Test.

TABLE 10. Simple correlation coefficients between mean alkaloid concentrations of leaf blades and total forage of 7 genotypes of reed canarygrass.

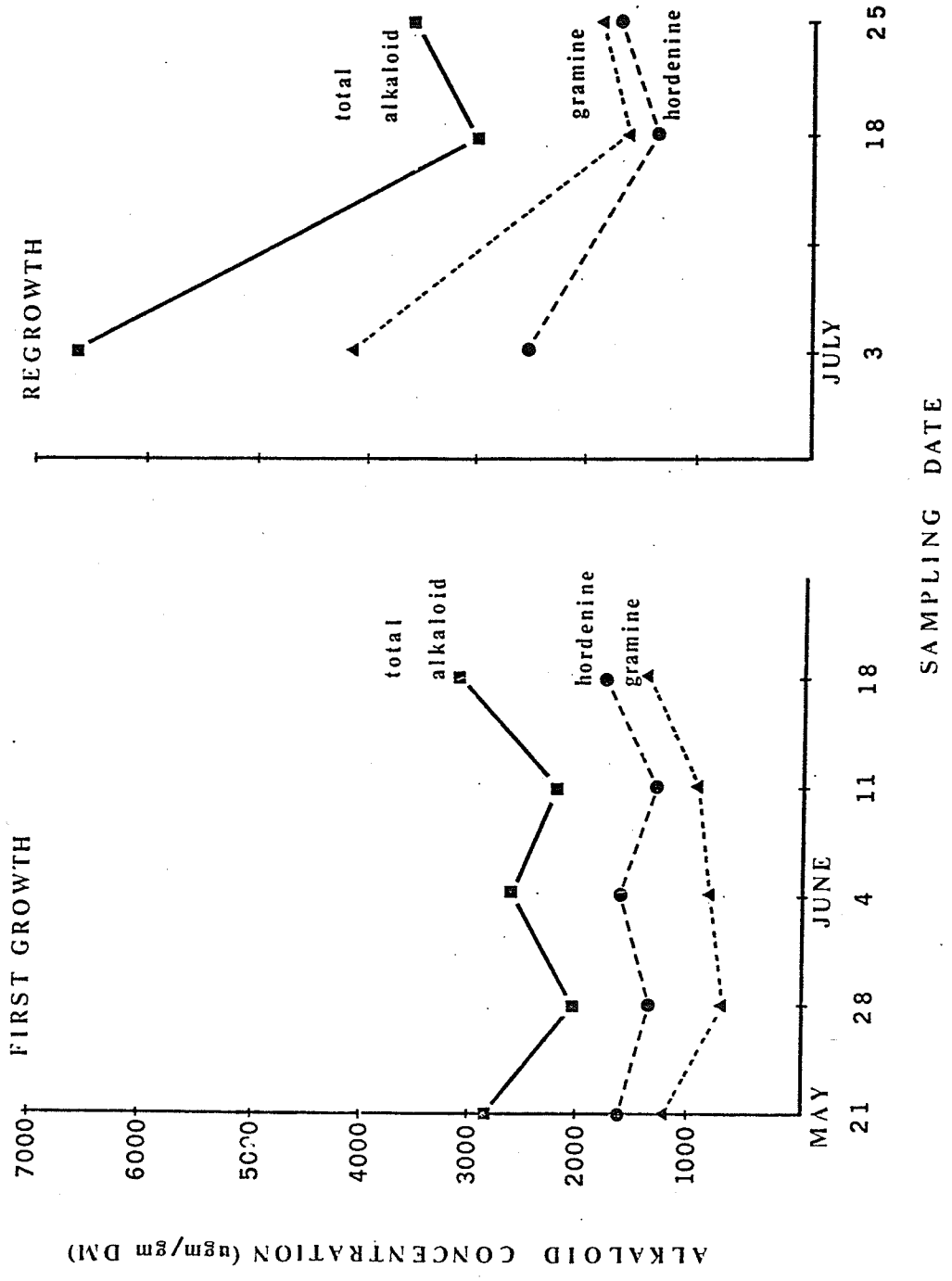
	Gramine	Hordenine
Upper Blade vs Total Forage	0.90 **	0.85 *
Entire Blade vs Total Forage	0.93 **	0.82 *

*, ** Significant p=0.05 and 0.01, respectively.

TABLE 11. Numbers of families, and ranges of parent and progeny means and overall means for gramine and hordenine concentrations (ugm/gm DM) in a greenhouse heritability experiment.

Alkaloid	No. of Families	Parents		Progenies		h^2
		Range	Mean	Range	Mean	
Gramine	35	80–1240	430	770–3240	1640	0.72
Hordenine	31	790–2380	1320	1180–6340	4500	0.53

FIGURE 1. Mean concentrations (ugm/gm DM) of gramine, hordenine and total alkaloids (gramine + hordenine) of leaf blades of first growth and regrowth reed canarygrass.


FIRST GROWTH
REGROWTH
total alkaloid
hordenine
gramine
7000
6000
5000
4000
3000
2000
1000
MAY
JUNE
JULY
21
28
4
11
18
3
18
25
ALKALOID CONCENTRATION (μgm/gm DM)
SAMPLING DATE

GENERAL DISCUSSION

DISCUSSION

The methods described in the present studies for the determination of gramine and hordenine concentrations should prove useful for routine screening in plant breeding programs. In our program, we found that 20 samples per day could be assayed in duplicate for both these alkaloids by one technician. The assays are fairly simple, requiring no particularly sophisticated equipment.

The assay for hordenine is somewhat of a problem, as the reactivity of the diazo reagent declines rapidly after preparation (see Appendix 2). The occurrence of this decline was not realized when the introduction nurseries were assayed for hordenine. Hordenine data from these nurseries were rejected and material was not available for repeat assays. This explains the absence of a major study of hordenine variability of reed canarygrass genotypes, such as that reported for gramine in the present study. The hordenine data that is included in the present manuscripts was obtained using small numbers of samples for each new preparation of the diazo mix. When assaying 20 samples per day, we divided them into 4 groups of 5. Thus, 5 duplicate samples and one blank were done with each fresh preparation. The diazo reagent could be dispensed into these samples within 30-45 seconds after preparation. Recently, D. Woods (personal communication) has

modified the assay to eliminate the problem of declining reactivity. This method involves the direct addition of sodium carbonate to the sample dissolved in ethanol. The mixture of ρ-nitro aniline and sodium nitrite is then added.

It is of interest to relate the alkaloid concentrations found in the present study to concentrations reported to cause animal problems. Jordan and Marten (1975) found almost no weight gain in ponies grazing a reed canarygrass pasture with a total alkaloid concentration of 0.32% (3200 ugm/gm DM). Earlier in the season, when the alkaloid concentration of the pasture was 0.10%, pony weight gains with reed canarygrass were similar to those found with bromegrass and orchardgrass. Marten et al. (1976) reported weight losses in steers grazing pastures with greater than 0.60% total alkaloid. The steers gained weight on pastures with alkaloid concentrations ranging from 0.07% - 0.32%. Gains were comparable on all pastures within this latter range. With lambs, gains were poor or losses occurred on pastures ranging from 0.22 - 0.40%. On pastures of 0.11% or less gains were much improved. Simons and Marten (1973) found pastures with less than 0.3% total alkaloids were fairly readily consumed by sheep, while those with greater than 0.8% were almost totally rejected.

From the above data, it is difficult to state a specific alkaloid concentration below which animal performance is not substantially affected. Knowledge of such a concentration would be useful as a goal for breeding programs. The presence of tryptamines and carbolines complicates the situation as animal

performance is poorer when these compounds are present. As well, the studies above do not include hordenine in the alkaloid concentrations reported. In any case, sheep seem to be the most sensitive to alkaloids and very poor performance has been found on pastures with greater than 0.2% total alkaloid. In the present study, many reed canarygrass lines were found to exceed 0.2% (2000 ugm/gm DM) gramine in regrowth with the 2 year mean being 0.215%. Since these genotypes were TC-, the gramine concentrations should be equivalent to the total alkaloid determinations reported above. There were also several lines with mean gramine concentrations less than 0.1% in regrowth. The variety Rise contained 0.288% while Grove was found to have 0.109% gramine in regrowth. In most lines, genotypes with concentrations less than 0.1% were found and we have isolated several of these to obtain seed for further studies.

In the present study, it was hoped plants free of gramine and perhaps also hordenine would be found and that these alkaloids would be as simply inherited as the tryptamines. In 1974, two plants were found in the introduction nurseries that were apparently free of gramine. In the greenhouse heritability experiment, several plants also appeared to be gramine free. Further sampling of regrowth material from these plants showed them all to contain low levels of gramine. We have not looked extensively at TC+ plants for the presence of gramine. A small number of plants have been screened by thin layer chromatography (Woods and Clark, 1971b) and these have all been gramine containing.

The opportunity to select strains of reed canarygrass low in alkaloid concentration appears promising. A wide variability for both gramine and hordenine was found in material included in the present study. Marten (1973) reviewed work on the reed canarygrass alkaloids and reported very wide ranges in total alkaloid concentrations over a number of studies. Alkaloid concentration in reed canarygrass also appears to be a highly heritable character. In the present study, gramine data for the introduction nurseries was highly correlated between years. Data from this study and also results from clones used in the *in vitro* digestion study were highly correlated between samplings. These results agree with the high correlations for total alkaloid data over different environments found by other workers (Marten *et al.*, 1973; Barker and Hovin, 1974; Hagman *et al.*, 1974). The narrow-sense heritability estimates for gramine (h^2=0.72) and hordenine (h^2=0.53) fall within the range of estimates for total alkaloid concentration determined by Barker and Hovin (1974). The lower narrow-sense heritability estimates for hordenine as compared to gramine corresponds to the lower correlation of hordenine data between harvests found for the clones in the *in vitro* digestion study.

Alkaloid data between field and greenhouse has been found to be highly correlated (Marten *et al.*, 1973; Hagman *et al.*, 1974). The present study has shown moderate heritability estimates for gramine and hordenine concentrations in reed canarygrass grown under greenhouse conditions. This suggests that selection for low alkaloid concentrations could be effectively done in the greenhouse.

Seedlings could be established in greenhouse flats during the winter. Initial screening would be for the presence of tryptamines and carbolines (Woods and Clark, 1971a). TC+ plants would be discarded and the remainder assayed for concentrations of gramine and hordenine. Plants with high concentrations of these alkaloids would also be discarded and the remainder transferred to a field nursery in spring. Thus, a nursery of TC- plants with lower concentrations of gramine and hordenine would be established and this could then be screened for other characters of interest. This would result in a considerable saving of field space. In the greenhouse screening, care would have to be taken to insure all plants were of similar maturity. The present study and others (Hagman et al., 1974) have shown a great variation in alkaloid content with maturity. The procedure described in the present study of trimming plants several times followed by fertilization should minimize differences in maturity caused by slow developing seedlings.

Hordenine has previously been reported to be a major alkaloid of reed canarygrass (Audette et al., 1969; Williams et al., 1971). The present study has shown it to be the most concentrated alkaloid of many genotypes (Appendix 3). The concentration of hordenine was found not to increase as dramatically as that of gramine in regrowth material. In the in vitro digestion study, the genotypes contained similar hordenine concentrations in both first growth and 6-week regrowth, while gramine concentrations in regrowth were more than double those of first growth. In the

study of alkaloid variation with maturity, hordenine concentrations of leaf blades were higher than gramine concentrations in first growth, but lower in regrowth.

Hordenine was distributed throughout the reed canarygrass plant with highest concentrations occurring in the leaf sheath. In the present study, and in other studies (Hagman et al., 1976), alkaloids were found to be most concentrated in leaf blades of reed canarygrass. It is usual to find alkaloids accumulating in plant tissues that are the most active in metabolism (Robinson, 1974). It is difficult to explain the deviation of hordenine from this general pattern as the function of hordenine, and in fact the function of most alkaloids in plants, remains obscure.

There has been no research done to examine the effect of hordenine on the grazing animal. Stecher et al. (1960) reported that hordenine has been used as a myocardial stimulant, and in small doses as an intestinal relaxant. In larger doses it is an intestinal stimulant. Perhaps hordenine has been involved in diarrhea and increased respiration rates seen in animals grazing reed canarygrass pastures. This alkaloid certainly warrants further research, especially since it is one of the major alkaloids of reed canarygrass.

SUMMARY & CONCLUSIONS

SUMMARY AND CONCLUSIONS

A number of reed canarygrass introductions and varieties were screened for alkaloids. Most strains contained genotypes free of tryptamines and carbolines. Among tryptamine-carboline free genotypes a wide range in concentrations of gramine and hordenine was found. Gramine and hordenine data were highly correlated between years and between harvests. The narrow sense heritability estimate for gramine was 0.72 and for hordenine, 0.53.

Highest concentrations of hordenine were found in the leaf sheath, while gramine was most concentrated in the leaf blade. Leaf blade concentrations of both alkaloids were highly correlated to those of the total herbage. Gramine and hordenine increased slightly in leaf blades of first growth reed canarygrass. New regrowth showed a great increase in these alkaloids, but in older regrowth, concentrations had fallen to those found in first growth.

Varying concentrations of gramine, hordenine, NMT and 5MeO-DMT added to <u>in vitro</u> fermentation media had no effect on IVD values. Alkaloid concentrations of selected reed canarygrass clones were not related to their IVD's.

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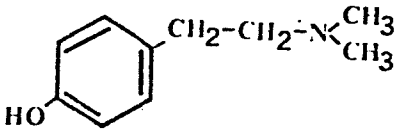
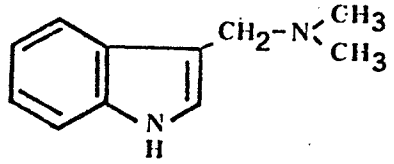
A P P E N D I C E S

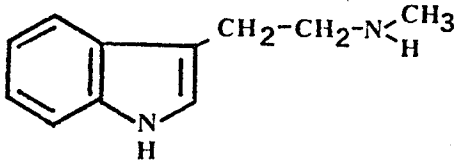
APPENDIX 1. Structure of alkaloids found in reed canarygrass.



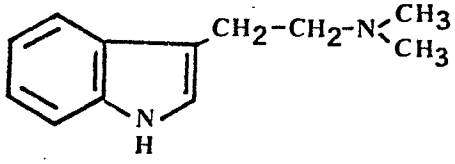
1) Hordenine



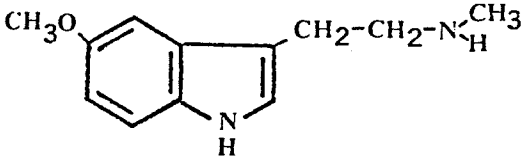
2) Gramine



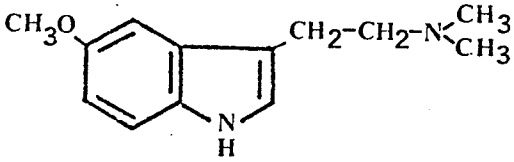
3) N-methyl tryptamine



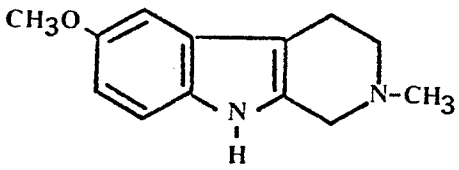
4) N,N-dimethyl tryptamine



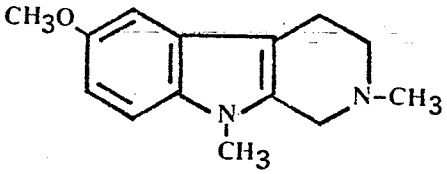
5) 5-methoxy-N-methyl tryptamine



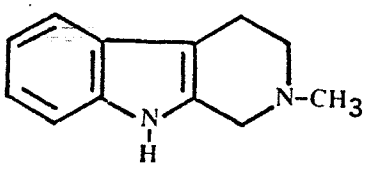
6) 5-methoxy-N,N-dimethyl tryptamine



7) 2-methyl-6-methoxy-1,2,3,4,-tetrahydro-β-carboline



8) 2,9-dimethyl-6-methoxy-tetrahydro-β-carboline



9) 2-methyl-tetrahydro-β-carboline

APPENDIX 2. Development of a method for extracting and assaying hordenine from reed canarygrass.

The following research which led to the development of an assay for hordenine was carried out in cooperation with Dr. D. Woods, as part of the total reed canarygrass program in the Plant Science Department, University of Manitoba

DETECTION OF HORDENINE BY THIN-LAYER CHROMATOGRAPHY

Pure samples of gramine (J. T. Baker Chem. Co.) dissolved in chloroform, and hordenine (Sigma Chem. Co.) dissolved in water were spotted on thin-layer chromatograms. Silica gel G (0.25 mm) TLC plates were used and the running solvent was methanol : concentrated ammonium hydroxide (7:1) as described by Woods and Clark (1971b). Several spray reagents were used to detect these compounds (Table 12).

Rf's for gramine were approximately 0.42 and for hordenine, 0.50. When varying concentrations of gramine and hordenine were used it was found that ferric chloride ($FeCl_3$) and Dragendorff's reagent were less sensitive than the others. Further work showed that Rf's for DMT and 5MeO-DMT were similar to the Rf for hordenine. Folin's reagent also produced a blue colour with these tryptamines.

EXTRACTION OF HORDENINE FROM PLANT MATERIAL

Extracts of reed canarygrass clones were made according to the procedure described by Woods and Clark (1971a). Extracts were concentrated and 10 ul spotted on TLC plates. No hordenine was

detected by the spray reagents listed in Table 12. However, gramine, and also tryptamines (in TC+ clones) were detected. The purification procedure used by Woods and Clark (1971a) involved basifying extracts with sodium hydroxide (NaOH) and re-extracting into chloroform. Since hordenine is a phenolic alkaloid, its sodium salt would be formed, causing it to be soluble in the aqueous phase and thus, discarded. This is in agreement with Manske (1950) who cautioned against the use of NaOH when extracting phenolic alkaloids. Concentrated ammonium hydroxide (3 ml) was substituted for NaOH in the purification procedure. Extracts from the above clones made using this procedure were all shown to contain hordenine.

COLORIMETRIC ASSAY FOR HORDENINE

The spray reagents produced a coloured product in combination with hordenine (Table 12). Thus, there existed the possibility of using one of these reagents in a quick colorimetric test tube assay for hordenine. Since the extraction procedure extracts all of the reed canarygrass alkaloids, the colour reagent should bind selectively with hordenine. The alkaloid extracts could be separated further by various forms of chromatography but this would not be feasible for screening large numbers of samples.

Dragendorff's and $FeCl_3$ were less sensitive than the other three reagents tested and thus, were not considered for the colorimetric assay. Folin's reagent produced the same colour of product with both hordenine and gramine. Xanthydrol produced a slowly developing yellow colour with hordenine, but this would be masked in a test tube by the pink colour produced with gramine. Diazotized ρ-nitro-aniline was

chosen as it produced an orange colour in combination with hordenine, but only a faint yellow with gramine. The absorption curves for the diazo reaction with gramine and hordenine are shown in Figure 2. The product formed with hordenine had a maximum absorbance at approximately 520 nm while the maximum for the gramine product was around 425 nm. The diazo reagent was much more sensitive for hordenine than gramine as the absorption curves were made with comparable concentrations of these alkaloids (i.e. 160 ugm hordenine, 185 ugm gramine).

The assay developed in the present study is based on the method described by Smith (1969) for the preparation of diazotized ρ-nitro-aniline. A diazonium ion is formed when $NaNO_2$ is added to ρ-nitro-aniline. This diazonium ion will then readily couple with aromatic amines or phenoxide ions to produce a coloured product known as an azo compound. The aromatic rings are joined by an -N=N-bridge.

Sodium carbonate was used in the assay since azo coupling occurs in basic conditions. The 5 ml of ethanol was added to clear the solution which is somewhat turbid after mixing the diazo reagent with the sample.

No differences were found in the OD of the azo compound when the ρ-nitro-aniline-$NaNO_2$ mixture was allowed to stand from 1 to 5 minutes before addition of the Na_2CO_3. After adding the Na_2CO_3, however, the reactivity of the diazo reagent declined rapidly with time. Diazo reagent that had been allowed to stand for varying time intervals after addition of Na_2CO_3 was added to samples of

213 ugm hordenine. The OD of the resulting azo compounds were: 0.65, when the diazo reagent had stood for 30 seconds; 0.36, for 90 seconds; and 0.14, for 180 seconds. Thus, for each preparation of diazo reagent, only a small number of samples (10 & 1 blank) were assayed. The diazo reagent could be dispensed into this number of samples within 30 seconds after the addition of the Na_2CO_3.

The calibration line for reference samples of hordenine is shown in Figure 3. The slope of the line was 0.0033. To determine extraction efficiency, reference samples of hordenine were dissolved in chloroform:methanol:concentrated ammonium hydroxide (26:33:1) and taken through the quantitative extraction and purification procedure. Extracts were assayed for hordenine and the % recovery calculated. An average of 34% of the hordenine was lost in the extraction procedure. The extraction efficiency of 66% was taken into consideration in the formula to calculate hordenine concentration.

TABLE 12. Spray reagents used to detect gramine and hordenine on TLC plates.

Spray Reagent	Preparation	Colour formed with: Gramine	Hordenine
Xanthydrol	0.1 gm in 100 ml ethanol: conc. HCl (95:5)	Pink	Yellow (develops after two days.)
Dragendorff's	A. Bismuth subnitrate (1.7 gm) in 100 ml HAc. B. Potassium iodide (40 gm) in 100 ml H_2O. Mix 50 ml A with 22 ml B.	Orange	Orange
Ferric Chloride ($FeCl_3$)	0.1 M solution	———	Gold
Folins	Folins reagent H_2O (1:1) followed by 20% (w/v) Na_2CO_3.	Blue	Blue
Diazotized ρ-nitro aniline	Solution of ρ-nitro-aniline (0.15 gm in 4.5 ml HCl + 95 ml H_2O) and $NaNO_2$ (5% w/v) followed by 20% (w/v) Na_2CO_3.	Faint Yellow	Orange

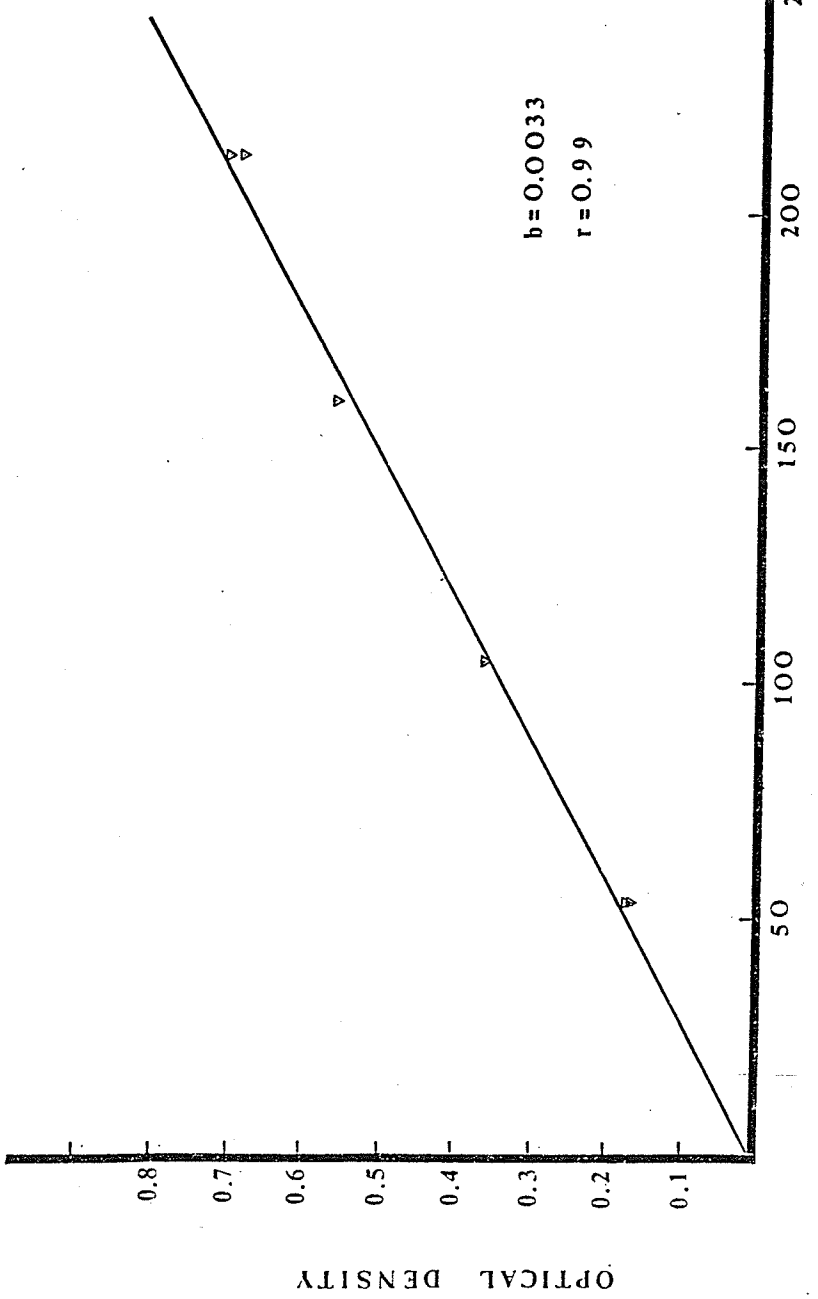


FIGURE 2. Calibration line showing relationship between hordenine concentration and optical density.

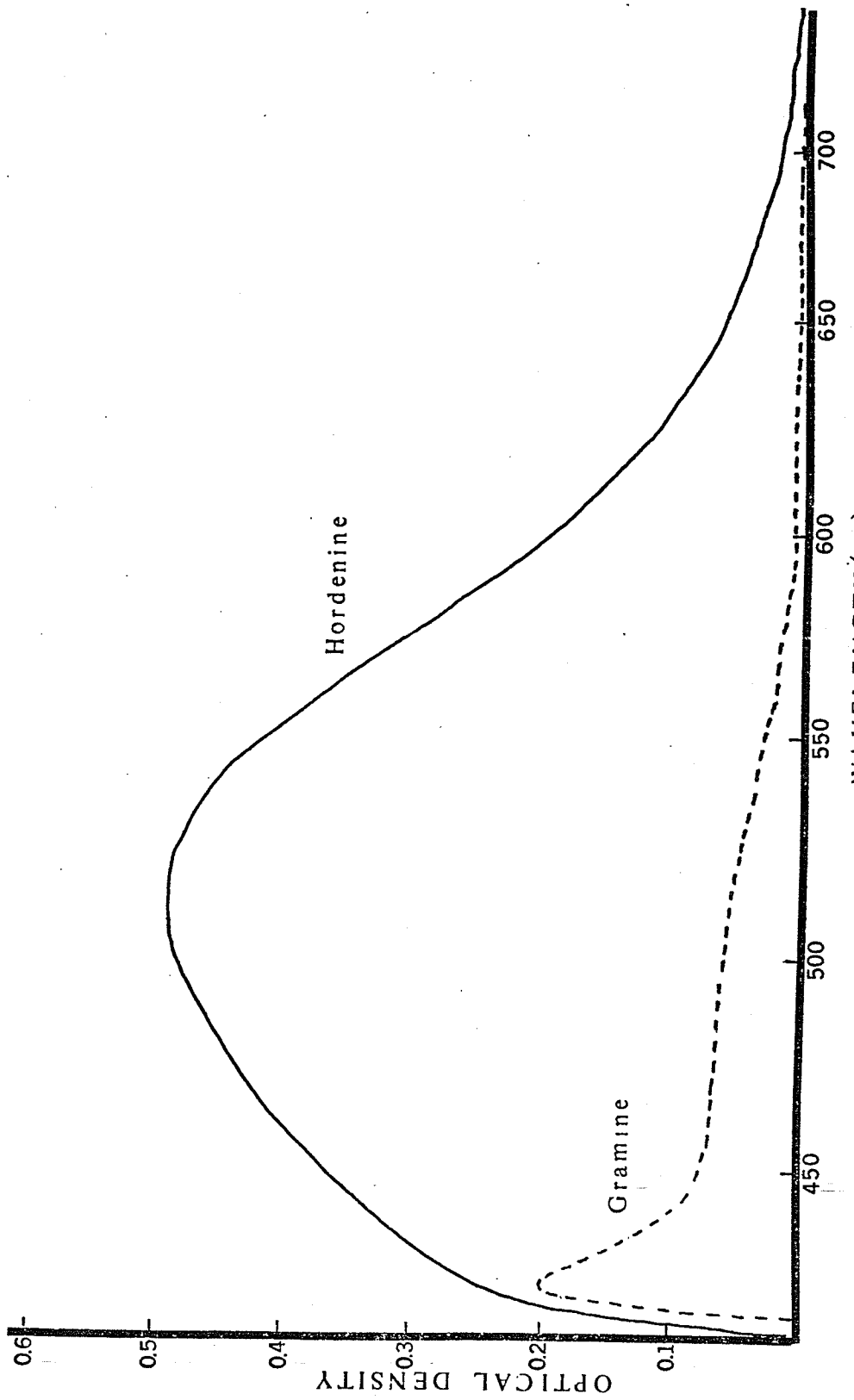


FIGURE 3. Absorption Curves of the Reaction Products of Diazotized p-Nitro-Aniline with Gramine and Hordenine.

APPENDIX 3. Concentration of hordenine in individual reed canarygrass genotypes.

Due to the initial flaw in the assay for hordenine, data from the introduction nurseries was rejected. Material was not available to repeat this study. In the manuscripts included in the present study, a number of genotypes were assayed for hordenine using the corrected method. Table 13 reports the hordenine concentrations found in these genotypes. Gramine data are included to serve as a comparison.

All but two of the nineteen diverse genotypes included in Table 13 contained greater concentrations of hordenine than gramine at the first cut. Mean hordenine concentrations were almost twice those for gramine at this stage. The four clones, for which samples of 2-week regrowth were taken, were all higher in gramine than hordenine. These samples were, however, of leaf blades only. Samples of 4-6 week regrowth had slightly higher mean hordenine concentrations.

Hordenine concentrations were probably much higher than those of gramine in first growth samples because the plants contained a higher proportion of stem. As well, a few of the samples had heads. Stem and heads are virtually devoid of gramine, but contain substantial amounts of hordenine. The four clones from which only leaf blades were sampled also contained somewhat more hordenine

than gramine in first growth. However, the concentration differences between the two alkaloids were not as marked as with the samples of entire forage.

The greenhouse heritability experiment of the present study also showed hordenine concentrations to be substantially higher than those of gramine. Concentrations of hordenine were approximately three times as high as gramine concentrations in both 2 and 4 week regrowth material. Samples were of the entire forage. The plants sampled in this study were derived from a topcross of clones of the cultivar Grove with S-6982, a seed retaining strain.

TABLE 13. Gramine and hordenine concentrations (ugm/gm DM) of first growth and regrowth samples of reed canarygrass genotypes.

Experiment	Clone	Geographic Origin	Type of Sample	1st Cut		2 Week Regrowth		4–6 Week Regrowth	
				Gram.	Hord.	Gram.	Hord.	Gram.	Hord.
Alkaloid Effects	18–34	Germany	Entire	2410	1850			3760	3060
on IVD	22–22	Switzerland	Forage	190	2220			570	1670
	2–39	New York		860	890			1860	1030
	6–24	Canada		120	1360			——	——
	22–15	Switzerland		230	1060			510	940
	30–11	Yugoslavia		150	810			530	1190
	30–23	Yugoslavia		140	820			330	660
	24–12	Austria		30	610			80	550
Alkaloid Variation	3–32	New York	Leaf	1080	1660	3590	2490	1110	1580
with Maturity	6–30	Canada	Blades	390	1010	2350	1500	550	770
	17–28	Germany		1100	1020	4500	2540	3060	1770
	22–42	Switzerland		1360	2300	6050	3450	2310	2020
Alkaloid	3– 4	Austria	Entire	180	930				
Concentration	3–32	Austria	Forage	130	1540				
of Plant Parts	6– 2	Canada		340	620				
	6–12	Canada		80	960				
	20– 8	Switzerland		250	1390				
	31– 3	U.S.S.R.		240	1440				
	31–10	U.S.S.R.		130	1650				
Mean				500	1270	4120	2500	1330	1390