1969

The Production and Behavior of Callus Tissue from Sorghum Vulgare

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THE PRODUCTION AND BEHAVIOR OF CALLUS TISSUE FROM SORGHUM VULGARE

BY

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A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Botany, South Dakota
State University

1969

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FROM SORGHUM VULGARE

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser _____ Date _____

Head _____ Date _____
Botany-Biology Department

ACKNOWLEDGEMENT

I would like to take this opportunity to thank Dr. David J. Holden for his very special guidance throughout this investigation. I also wish to express my appreciation to all the members of the Botany-Biology Department for their assistance and interest in this project. Special appreciation is expressed to the members of the Crops Research Division, U.S.D.A., Metabolism and Radiation Research Laboratory at Fargo, North Dakota, for their suggestions and interest while employed there as a summer trainee.

VJM

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LIST OF ABBREVIATIONS

Abbreviation	Term
FAA	formalin-acetic acid-alcohol
FeEDTA	iron ethylenediamine - tetraacetic acid
IAA	indole-3-acetic acid
IKI	potassium-iodide-iodine solution
GA_3	gibberellic acid
NAA	alpha-naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid

INTRODUCTION

The history of plant tissue culture dates back to the early 1900's with the research of Gottleib Haberlandt in Germany. However, it was not until 1939 that any real success was achieved. Three independent scientists, Gautheret, Nobecourt, and White simultaneously through their work set the pace for plant tissue culture.

Plant tissue culture is the isolation of cells or tissues from their association with other cells and tissues in the plant to one in which nutrients are supplied artificially and the cell or tissue acts as an individual. Once the plant cells or tissues have been studied at the individual level, more information about plant functions can be obtained.

Most tissue culture research has involved cells or tissues taken from many species of dicotyledonous plants but from very few monocotyledons. This thesis reports an attempt to initiate and grow callus tissue of the monocotyledon, sorghum. The objectives of the research were the following: to determine the conditions necessary to produce callus; to describe the morphology of callus origin and development; and to reproduce the results obtained with dicotyledons, the totipotency of cells (Sun 1966). To the author's knowledge, no research to date has been published on the tissue culture of sorghum.

The literature reviewed has been divided into four areas of monocotyledonous tissue culture: root culture, shoot and embryo

culture, endosperm culture, and callus culture. Some general references helpful to persons interested in plant tissue culture are Butenko 1968, Carew and Staba 1965, Willmer (ed.) 1966, Tulecke 1961, and White 1963.

Root Culture

As early as 1932, P. R. White considered the culture of excised root tips part of the field of tissue culture which at that time was being done primarily with animal tissues. White (1932) used wheat root tips excised from seeds which had been surface sterilized with calcium hypochlorite while continually rotated at about 60 rpm for 3 to 5 hours. The wheat root tip explant was 3 mm long and was placed in a 125 ml Erlenmeyer flask with 50 mls of liquid medium. The nutrient solution was that of Uspenski and Uspenskaia plus additions of 20 g/l dextrose and yeast extract prepared from 0.5 g of dried brewer's yeast. Four hundred simultaneous cultures growing for a period of 2 weeks were used to constitute a series. The best growth obtained, 266 mm or 19 mm/day, was on 25 mls of a medium consisting of a salts solution plus 20 g/l dextrose, yeast extract from 0.1 g of yeast for each liter of solution, and a pH of about 5 with temperature about 26° to 27° C and continuous light.

After P. R. White's pioneer attempts, W. J. Robbins and V. B. White reported in 1936 on the limited growth of excised corn root tips and in 1937 on the effect of corn extracts on the growth of excised root tips. They concluded that the abnormalities and

poor growth of corn root tips was due to the nutrient medium. Then in 1937 they incorporated the extracts from corn into the nutrient medium and tested the growth of corn root tips. Milk from immature grains was injurious while the diffusate from germinated corn grains benefited the growth of root tips on an agar medium of mineral salts and dextrose. The water extracts from the terminal portions of seedling corn roots at a low concentration favored root growth but at high concentrations injured corn root tips.

Finally, in 1950 Almestrand reported on his work with isolated roots of barley and oats. He pointed out the fact that roots of monocotyledonous plants at that time had not been grown indefinitely in a culture medium and that the reason was a cessation of growth caused by inactivity of the apical root meristem. Almestrand hypothesized that in the endosperm and leaves of monocots an unknown substance is synthesized which is necessary for the meristematic cell divisions of the root tip. Peptone was the only additional substance that gave any results with barley but was still not completely adequate for meristematic growth.

In a later work with cereal roots, Almestrand (1957) stated that the problem with isolated roots was that they had to take in sugar through their epidermis rather than getting sugar from leaves via the phloem. Roots that are unable to grow may not take in enough sugar. Almestrand found that roots generally did better on solid media than in liquid and that increasing the oxygen concentration by bubbling or shaking did not increase the growth of wheat, rye,

and oats. Continued growth of rye roots was obtained using yeast extract, while barley, oats, and wheat showed negative results. Coconut milk also inhibited the growth of barley, oats, and wheat roots.

E. H. Roberts and H. E. Street in 1955 suggested that the cessation of growth in culture of rye roots and roots of Gramineae in general is due either to 1. a requirement for some growth factor(s) or metabolite(s) not supplied by the root culture medium or 2. to a derangement of root metabolism resulting from detachment from the shoot and causing the accumulation of lethal substances in the root. The latter suggestion is similar to Almestrand's (1950) hypothesis, and both imply that there is some influence of the shoot which can never be supplied by the culture medium. Roberts and Street (1955) were successful in establishing clones from about only 10% of the grains of rye used and failed to establish excised root clones from other cereals tested. Their culture method was similar to that of White's (1932) except 12 mm root tips were used instead of 3 mm and a modified White's medium was used. Roberts and Street (1955) started clones by adding only yeast extract to White's medium; it was found that 80% of the growth stimulation by the yeast extract was due to its content of L-tryptophane.

It was not until the 1960's, some 30 years after P. R. White's (1932) first attempt at tissue culture of wheat root tips, that H. E. Street et. al. (1961) and J. D. Ferguson (1963) reported on the culture of excised wheat roots. A modified White's medium was

used in both cases. Street et. al. (1961) demonstrated that light, tryptophane and nitrate enhanced root growth. Ferguson (1963) used wheat root explants 10 mm long, 2% glucose, FeEDTA instead of ferric chloride, and 2.17 ppm L-tryptophane. Some substances found by Ferguson (1963) that substitute for L-tryptophane are D-tryptophane, indole, tryptamine, and IAA among others. Ferguson concludes that none of the wheat varieties tested over the last 30 to 40 years have provided culturable roots but suggests that the requirements of more easily cultured monocot roots will be found to differ little from their dicot counterparts.

In summary, tissue culture of monocotyledonous roots was first tried in 1932 by P. R. White with wheat root tips. His tissue culture methods became standard procedure for later workers with root culture and plant tissue culture as a whole. White (1932) obtained a good growth rate with wheat roots for a two week period by adding dextrose and yeast extract to the nutrient solution. A few years later, Robbins and White (1936, 1937) grew corn roots without much success on nutrient medium with corn extracts added. In the 1950's Almestrand (1950, 1957) worked with roots of cereals and was unsuccessful with roots of barley, wheat, and oats while continued growth of rye roots was possible only after the addition of yeast extract. Roberts and Street (1955) established clones of rye root tips from a small percentage of the root cultures tried but made an important discovery that L-tryptophane was responsible for much of the growth stimulation from yeast extract. Street et. al. (1961) and

Ferguson (1963) attempted the culture of wheat root tips but this time on a chemically defined media. Although the continuous culture of monocot roots has not been fully realized, many guidelines and discoveries have been made since White's first attempt. Scientists in this field still believe that once the requirements for monocot root culture are found, they will be basically similar to those of dicot root culture.

Shoot and Embryo Culture

Culture of monocotyledon shoots or embryos was undertaken some time later than the culture of monocot roots, possibly due to the difficulty of continuous root culture. A. J. Haagen-Smit et. al. (1945) tried the culture of excised immature corn embryos by using the methods for carrot culture. Corn embryos excised 8 to 10 days after pollination were 0.3 to 3 mm long but could not survive on the growth factors from coconut milk.

Shih-Wei Loo (1945) was able to grow asparagus stem tips through successive transfers on a medium of low salts developed by Bonner for isolated roots. Some compounds that promoted growth for several transfers were succinic acid, aspartic acid, and ammonium sulfate. Loo found that high sugar concentrations (8%) favored growth in the dark while low concentrations (1%) favored growth in the light. He proposed that there are two groups of growth substances produced naturally: one in the stem and one in the root.

After the not too successful attempts to culture corn embryos and asparagus stem tips, N. K. Ziebur et. al. (1950) attempted to

culture barley embryos. Ziebur et. al. found that immature embryos grown on 2% sucrose, minerals, and agar failed to complete the later stages of normal embryonic development but instead germinated into weak seedlings. The addition of 1% casein hydrolysate inhibited germination and prolonged embryonic growth. The inhibition was thought to be due to the high osmotic pressure of casein hydrolysate.

Barley embryos were later used extensively by K. J. Norstog. Norstog (1956) was concerned with the growth of barley embryos on White's medium plus Seitz filtered coconut milk. If coconut milk was autoclaved, the root development of 0.15 mm embryos was inhibited. On 20% coconut milk the embryos callused, but grew normally only on 90% coconut milk. In later work, Norstog (1963, 1967) defined a modified White's medium for the growth of very small (200-400 μ) barley embryos. This medium was high in phosphate, was supplemented with 11 amino acids and contained 9% sucrose. Norstog's objective was to eventually culture zygotes in order to study poles and planes of symmetry as well as orientation of the embryo in the ovule.

Possibly the latest report on the culture of monocot shoots or embryos is that by C. W. Smith in 1967. Smith (1967) studied the growth of excised embryo shoot apices of wheat but was not too successful.

In summary, less work has been done in the area of monocot shoot or embryo culture compared to that done with root culture. Initial attempts at embryo culture with corn, following methods used with carrot and by adding coconut milk, failed. Embryos of barley

were chosen for intensive study by K. J. Norstog (1956, 1963, 1967), who was able to chemically define a culture medium that would support the growth of very small embryos. In the area of shoot culture, best results were obtained by Loo (1945) who used asparagus stem tips and who also suggested as did Almestrand (1950) and Roberts and Street (1955) that a difference exists in growth substance concentration and metabolism between shoot tips and root tips.

Endosperm Culture

C. D. LaRue (1949) was the first to accomplish the continuous culture of corn endosperm. In 1954, J. Straus and LaRue (1954) listed the culture requirements for maize endosperm grown *in vitro* as being modified White's medium containing 2% sucrose and 0.5% Seitz filter-sterilized yeast extract. Straus (1960) reported the development of a synthetic medium. It was found by paper chromatography of fresh tomato juice, which could also produce growth equal to yeast extract, that tomato juice contained a large amount of free amino acids. The constituent in the tomato juice that provided as good as or better growth than any complex organic supplement was asparagine at 1.5×10^{-2}M. The addition then of asparagine to the mineral, sugar, and vitamin medium gave a chemically defined medium.

T. Tamaoki and A. J. Ullstrup (1958), two years before Straus (1960) had defined a medium for endosperm culture, reported on the cultivation of excised endosperm and meristem tissue of corn. Modified Nitsch's medium supplemented with yeast extract was used for sugary endosperm excised 8 to 11 days after pollination. The

friable, active growth of endosperm tissue made it appear to be able to grow indefinitely. Cultures on yeast extract medium did not respond to IAA, other growth substances, or casein hydrolysate at the concentrations used. Coconut milk did not replace yeast extract. Gautheret's medium was the only one that induced proliferation of meristem explants. With explants of either node or internode tissue no cell divisions were observed, but when the explant contained more than 2 nodes normal growth like that of an excised shoot was observed. No growth was observed in liquid culture.

Of other monocot endosperm tissues grown in culture, some success with rye-grass endosperm was achieved by K. J. Norstog (1956). Twenty per cent of the endosperms produced tissue masses large enough for subculture in five days when White's medium, 2% sucrose, 1 mg/l IAA, 20% Seitz-filtered coconut milk, and 0.25% yeast extract were used. Best results were obtained at pH 6.1 and at 26° C.

The culture of endosperm tissue of monocots, similar to that of shoot, embryo, or root culture, has relied upon the organic compounds found in such complex supplements as yeast extract, coconut milk, tomato juice, casein hydrolysate, and others. Only recently (Straus 1960) a synthetic medium has been defined that will support the growth of a variety of a sugary corn endosperm. Endosperm tissue might represent a relatively undifferentiated tissue compared to whole shoot or root organs and therefore be more easily culturable; although Norstog (1956) recognized the general absence of callusing

in monocots either *in vivo* or *in vitro*. Endosperm culture, which is not a true callus culture, is still important for the study of storage products, enzyme interactions, and unusual chromosome numbers.

Callus Culture

A. W. Galston (1948) in response to Loo (1945), found that excised asparagus stem tips often produced callus when grown on the same medium used by Loo plus 2% sucrose and 10 λ/cc or higher IAA concentrations. This callus initiated by IAA on asparagus stem tips grown in the dark later gave rise to an extensive root system and a new shoot. In the light under the same concentrations, no callus was initiated. Galston's (1948) observation is possibly the first account of callus initiation from a monocotyledon.

In 1951, the classic paper of G. Morel and R. A. Wetmore was published on the "Tissue culture of monocotyledons". Morel and Wetmore (1951) claim to be the first to successfully obtain a callus or to culture root tissue from monocots. The authors used a tropical member of the *Araceae* family which produces a large underground tuber weighing several pounds. The tuber was sterilized for 20 minutes in 5% calcium hypochlorite. NAA at concentrations of 10^{-6}, 10^{-7}, and 10^{-8}M and the B vitamins when added to the culture medium gave good callus growth for two months but then growth decreased. The callus was then transferred to a medium in which the B vitamins had been replaced by 15% coconut milk from green coconuts. Green coconut milk seemed to give good growth again, but the same medium containing 15%

ripe coconut milk did not promote active callus growth. The callus obtained from the tuber tissue had a brownish look and was made up of both organized growth in the form of roots and buds and unorganized growth or callus. Roots were more abundant at high concentrations of auxin.

After this classic work Norstog (1956), while working on the growth of barley embryos, reported that embryos formed callus on 20% coconut milk. D. P. Carew and A. E. Schwarting then in 1957-58 reported on the production of callus from rye embryos. Carew and Schwarting (1957-58) stated that there were very few monocotyledons from which callus tissue capable of prolonged cultivation had been obtained at that time. These authors tried three types of tissues, nodal areas of young stems, endosperm tissue of various ages, and 15 to 20 day old embryos. From the first tissue a callus, which could not be successfully subcultured, was produced in one instance; no callus was produced in all cases tried from the second tissue; but abundant callus was produced from embryos. A modified Heller's medium containing 10 vitamins, 2% sucrose, 0.5% yeast extract and 1 mg/l 2,4-D gave the most rapid (8 days) callus growth at a pH 5.8 and 26° C either in the dark or in the light. Coconut milk (15%) plus modified Heller's medium gave callus in 15 days. IAA was inferior to 2,4-D; kinetin, GA_3, and adenine sulfate had no effect.

One step closer to indefinite growth of callus tissue from monocots was achieved by Mascarenhas et. al. (1965) working with Zea Mays. Although the callus tissue from maize seedlings, variety

Golden Bantam, was grown over 2 years through continuous subculture, a true homogeneous callus state was never reached. The authors reported that root formation occurred in almost all cultures which indicates only a partial dedifferentiation. Five mm fragments from stem and root portions of 7 to 8 day old seedlings were placed on White's medium, pH 5.8, supplemented with NAA (1 ppm) and either corn steep liquor, CSL, (0.2%), sym-diphenyl urea, DPU, (1 ppm) or edamine-S (1.1%). 2,4-D (0.6 ppm) was inhibitory, and tissue turned black after a few days. Mascarenhas et. al. (1965) found that their callus tissue had an absolute requirement for DPU; DPU has been reported to be a constituent of coconut milk. When only the callus was transferred to liquid medium, roots formed but only in the presence of CSL or DPU.

Not until about 10 years after Carew and Schwarting (1957-58), J. M. Webster (1966) described the production of oat callus on Heller's medium. Webster inoculated the whole germinated seed on a basal medium containing 2,4-D at 5 mg/l, IAA at 2 mg/l, and glucose at 2%. In about 6 weeks this medium produced a firm, golden oat callus which was maintained through several subcultures for three years. Callus formed more readily in light than in dark and at a temperature of 24°C. Callus was also initiated on a medium containing sucrose, 2,4-D and EDTA; poor callus growth occurred on sucrose plus IAA and NAA, and on sucrose plus 2,4-D; no callus was obtained on sucrose only or sucrose plus casein hydrolysate and yeast extract.

O. Carter et. al. (1967) only a short time after Webster's (1966) report, stated that to their knowledge no one had reported the successful induction and growth of oats callus. Carter et. al. (1967) used Linsmaier and Skoog's (1965) medium except no optional constituents were added. Callus first formed from the root system; the shoot remained alive for 2 months but eventually died. The callus growth continued and has been subcultured. At low concentrations of auxin, callus developed from the meristematic zone; at greater concentrations the whole root system was completely replaced by callus with the initial callus forming at the juncture between roots and coleoptile. The authors conclude that callus induction is very dependent on concentration of auxin but kinetin is of little importance. Callus was initiated by IAA but only at much higher concentrations than the 2,4-D concentrations. The 2,4-D concentrations ranged from 22.5 to 225 μmoles/l.

As Webster (1966) and Carter et. al. (1967) successfully cultured oat callus, Yatazwa et. al. (1967) was culturing rice. Yatazwa et. al. (1967) did a standard experiment with callus derived from roots of 5-day old rice seedlings growing on a modified Heller's medium containing 2,4-D and yeast extract. 2,4-D at a concentration of 2 ppm was necessary to initiate callus from rice roots, while a concentration of 0.05 ppm provided optimum growth of callus explants. IAA was ineffective at initiating callus even at 100 ppm. Yeast extract (0.5%) was found to be essential for indefinite growth even though the B vitamins and others were present in the basal medium.

The optimum concentration of sucrose was 3-4%; glucose, fructose, and maltose supported growth equally well. Xylose, starch, and glycerol among others supported growth poorly or not at all. The optimum initial pH was between 5 and 6 while the optimum temperature for growth of callus was between 25° to 30°C, which is somewhat higher than that for other plant callus tissue. No organ differentiation occurred even on medium without 2,4-D; some vascularization and lysigenous lacunae formation was observed.

C. Wilmar and M. Hellendoorn (1968) some 20 years after Galston's (1948) work with asparagus stem tips, were able to produce embryoids and eventually plants from asparagus cells grown in vitro. The stock callus was derived from hypocotyl tissue of seedlings grown on Linsmaier and Skoog's (1965) basal medium with 2,4-D (1 mg/l) and kinetin (0.315 mg/l) at 25° ± 2°C under continuous illumination. Green callus was produced on this medium with some organized growth, but when 2,4-D and kinetin were omitted many shoots with few or no roots were formed. The callus was then placed in suspension culture and rotated at 100 rpm. In suspension culture medium with the same concentrations of 2,4-D and kinetin used for callus initiation, callus growth was rapid at first then changed to many small, round cells which aggregated and formed 50 to 200 μ nodules. They found that with decreasing concentrations of 2,4-D, cell clumping and embryoid formation increased. Kinetin was beneficial for embryogenesis but was only needed at low concentrations (0.1 mg/l). Only small embryoids, less than 2 mm, grew into normal plants, and these when

grown on solid medium of the same composition as that of liquid tended to dedifferentiate again.

The *in vitro* culture of somatic wheat callus tissue by E. J. Trione *et. al.* (1968) is one of the latest accounts of monocot tissue culture. Twenty different media were tested but best callus growth was supported by Hildebrandt's "D" medium with chelated Fe instead of Fe-tartrate. Callus was obtained from wheat tissue excised just below the cotyledonary node of germinating wheat seeds and also from apical portions of the larger roots. Callus growth rate was slow compared to that of dicots such as tobacco and grew better on solid rather than liquid medium. By omitting 2,4-D and increasing NAA from 0.1 to 10 mg/l, wheat callus differentiated many roots either in liquid or solid Torrey and Reinerts or Hildebrandt's "D" media. Wheat root growth on these new media compared to that used by earlier researchers with wheat roots is speculated by the authors to be useful in studying the physiology of a wheat-root system. Shoot primordia or leaves were not seen in any callus cultures of any of the 13 wheat varieties tested.

Callus culture in contrast with root, shoot, or endosperm culture of monocotyledons has only recently come into being, with five out of eight articles in this area written in the last two years. Plants from which callus cultures have been obtained are limited to only a few members of the *Graminaea* such as rye, corn, barley, rice, oats, and wheat and to other monocots like asparagus and a tropical family, *Araceae*. Callus culture of monocots has posed problems

unique to plant tissue culture e.g., requirement for relatively high concentrations of growth regulators such as 2,4-D and lack of ability to differentiate into organized growth. Monocot callus culture still requires some exotic organic supplement, usually coconut milk or yeast extract for indefinite growth. Two culture media, modified Heller's and Linsmaier and Skoog's, have been used regularly for monocot callus culture. Other general requirements seem to be 2% sucrose, coconut milk (10-15%) or yeast extract, a pH between 5 and 6, and temperature between 23° and 30°C. Whole plant or organ regeneration from monocot callus tissue has not been realized from most tissues cultured to date except for that reported by Wilmar and Hellendoorn (1968) with asparagus.

METHODS AND MATERIALS

Grain sorghum, Sorghum vulgare, was the plant species used in this research. Two varieties of grain sorghum, Norghum harvested in 1964 and North Dakota No. 104 harvested in 1966, were tested. Norghum was the variety used most extensively and unless it is stated otherwise, it will be assumed the results obtained were with Norghum. All seeds were surface sterilized with either a 2% solution by weight of calcium hypochlorite or a 10% by volume solution of Clorox. Sterilization time ranged from 10 to 30 minutes with the seeds and solution stirred continually by a magnetic stirrer. The sterile seeds were placed aseptically onto moistened filter paper in sterile petri dishes and germinated from 5 to 7 days in the dark at room temperatures. All transfers and inoculations were carried out in a transfer chamber with positive air pressure and an ultraviolet germicidal lamp. A bactericidal detergent for washing walls of the transfer chamber, an alcohol lamp for flaming instruments and 70% ethanol for disinfecting instruments were materials used to help reduce contamination. Glassware, media, and instruments were autoclaved for 20 minutes at a pressure of 15 to 18 lbs/sq inch and 120°C.

Murashige and Skoog's (1962) Revised Medium 1962, RM62, culture medium was used throughout this research. The RM62 basal medium consists of macro-inorganic and micro-inorganic salts, vitamins, sucrose, and agar. The first three ingredients were prepared from

stock molar solutions; common table sugar was used for sucrose. An Agar Agar No. 3 pill supplied enough agar for 40 mls of culture medium in a 125 ml Erlenmeyer flask. IAA and 2,4-D were prepared on a ppm basis and kept refrigerated. Coconut milk was obtained from coconuts purchased at local grocery stores by draining the coconut, boiling the juice, and filtering. All supplements to the basal medium were added before autoclaving. The pH of the culture medium was adjusted with dilute HCl or KOH to 5.6-5.8 before autoclaving.

Both liquid and solid culture media were used in this research. Erlenmeyer flasks (125 ml) contained 40 mls of agar medium; T-tubes contained 25 mls of liquid medium and the T-tubes were shaken on a reciprocal shaker or a rotating shaker (Fig. 1). Most experiments were carried out in diffuse light at room temperatures of $23^{\circ} \pm 2^{\circ}C$.

Plant tissues for anatomical studies (Fig. 4) were fixed in FAA, imbedded in paraffin, sectioned at 8 to 10 μ on the rotary microtome and stained with safranin and fast green. Fresh tissue was frozen and sectioned with a sliding microtome, stained with IKI to distinguish starch grains, and temporarily mounted for observation (Fig. 9).

A photographic procedure was used for recording the qualitative growth of plant tissue. A 35 mm camera with extension tubes and colored film was used to take closeup pictures through the culture flask with a magnification of approximately one half times. Photomicrographs were taken either with phase contrast or bright field

microscopy.

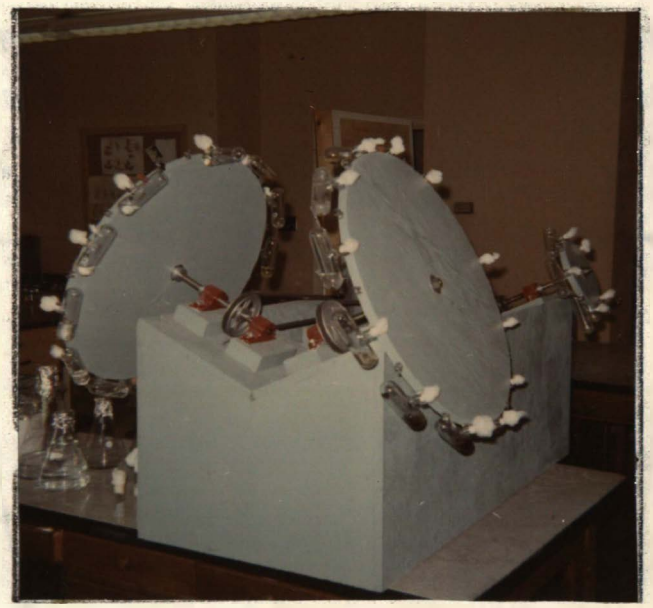


Figure 1. Rotary shaker built especially to hold liquid culture T-tubes, speeds: 4 rpm large wheel, 10 rpm small wheel.

RESULTS

The effect of 2,4-D concentration in ppm on the germination, shoot length, and root length of sorghum seeds, variety N.D. 104, is shown in Fig. 2. The data plotted is that taken from an average of 20 seeds (two replications of 10 seeds for each concentration tested). The experiment was run twice, once with Norghum and once with N.D. 104; similar results were obtained. Germination time was from 6 to 7 days.

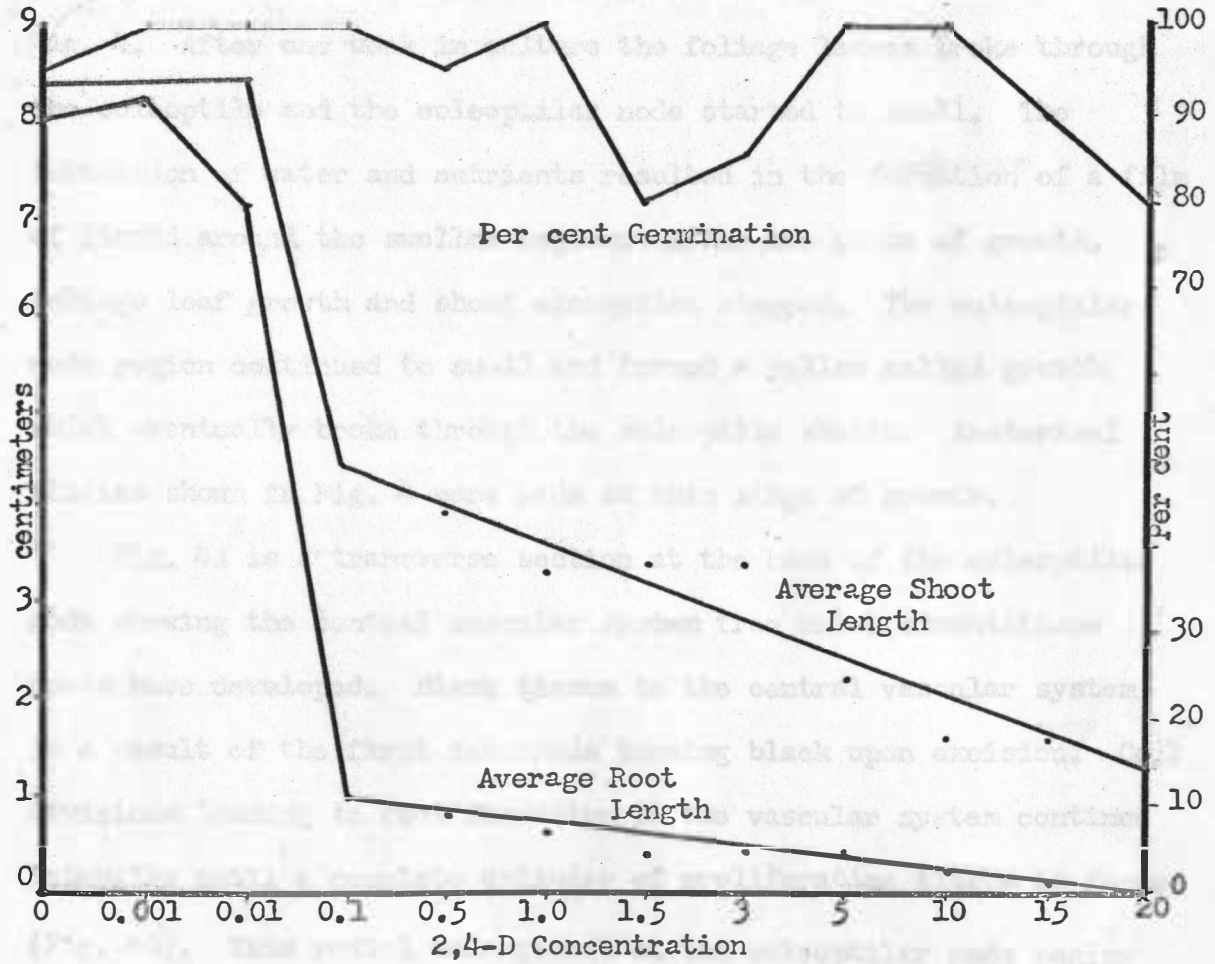
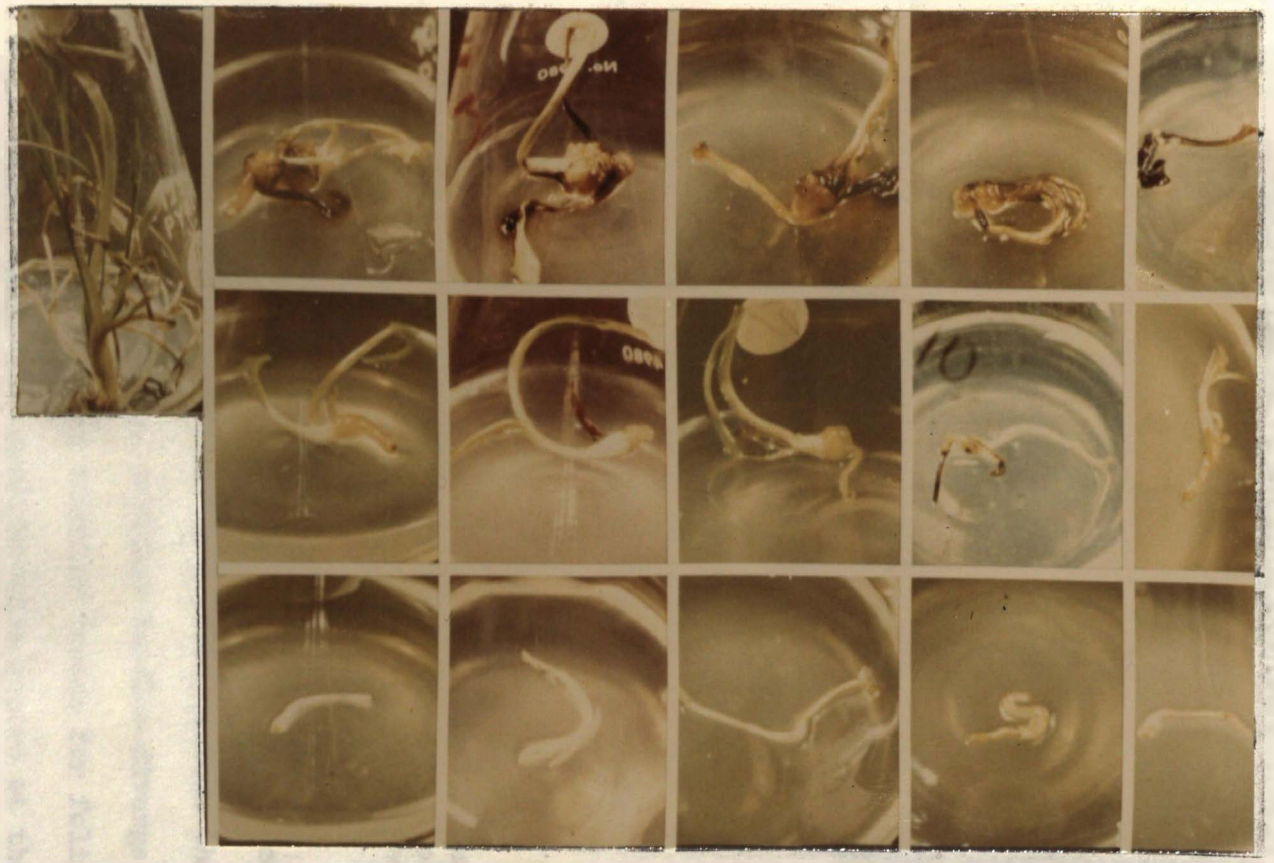


Figure 2. The effect of 2,4-D on the germination, shoot, and root length of sorghum seedlings.

The initiation of callus in sorghum shoots, Fig. 3, was tried at 2,4-D concentrations of 0, 1, 3, 7, 10, and 15 ppm over a time period of two months. Shoots from seedlings germinated 7 days were excised just below the coleoptile and grown on an agar medium of RM62 supplemented with coconut milk (10%) and sucrose (2,5%). Pictures were taken of representative samples from 10 replications of each concentration tested, Fig. 3.

Callus initiation from sorghum shoots grown on RM62 basal medium plus 2,4-D and 10% coconut milk (Fig. 3) took place as shown in Fig. 4. After one week in culture the foliage leaves broke through the coleoptile and the coleoptilar node started to swell. The imbibition of water and nutrients resulted in the formation of a film of liquid around the swollen region. After two weeks of growth, foliage leaf growth and shoot elongation stopped. The coleoptilar node region continued to swell and formed a yellow callus growth which eventually broke through the coleoptile sheath. Anatomical studies shown in Fig. 4 were made at this stage of growth.

Fig. 4A is a transverse section at the base of the coleoptilar node showing the central vascular system from which adventitious roots have developed. Black tissue in the central vascular system is a result of the first internode turning black upon excision. Cell divisions leading to root formation in the vascular system continue laterally until a complete cylinder of proliferating tissue is formed (Fig. 4B). This radial enlargement at the coleoptilar node region continues upward into the short internode by increased periclinal



Control 1 ppm 3 ppm 7 ppm 10 ppm 15 ppm

Figure 3. Callus initiation in sorghum shoots. Effect of increasing 2,4-D concentration at 1 week, 3 weeks, and 2 months of growth on RM62 and coconut milk (10%) and sucrose (2.5%). Note the pigmentation of shoot and discoloring of medium at 2 months.

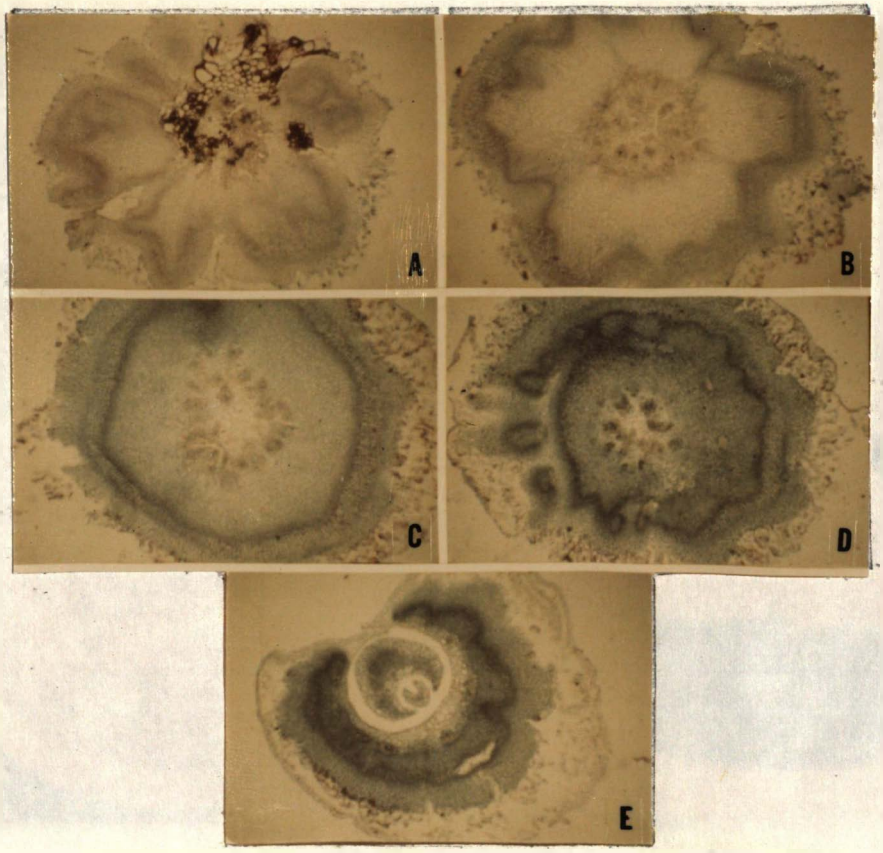


Figure 4. Callus formation in young sorghum shoots. (A) area just below coleoptilar node (B) coleoptilar node (C) area of second internode (D) second node (E) area above second node. Magnification X43

divisions of parenchyma cells (Fig. 4C). The process repeats itself again in the second node but to a lesser extent than at the node of the coleoptile. Finally, vascular bundles diverge (Fig. 4C) from the vascular plate and become vascular traces for foliage leaves (Fig. 4D,E). Some cell proliferation occurs at the periphery of leaf sheath bases attached to the second node (Fig. 4D,E).

Primary callus tissue of the type shown in Fig. 5A and Fig. 3 at 2,4-D concentrations of 1, 3, or 7 ppm was transferred to fresh

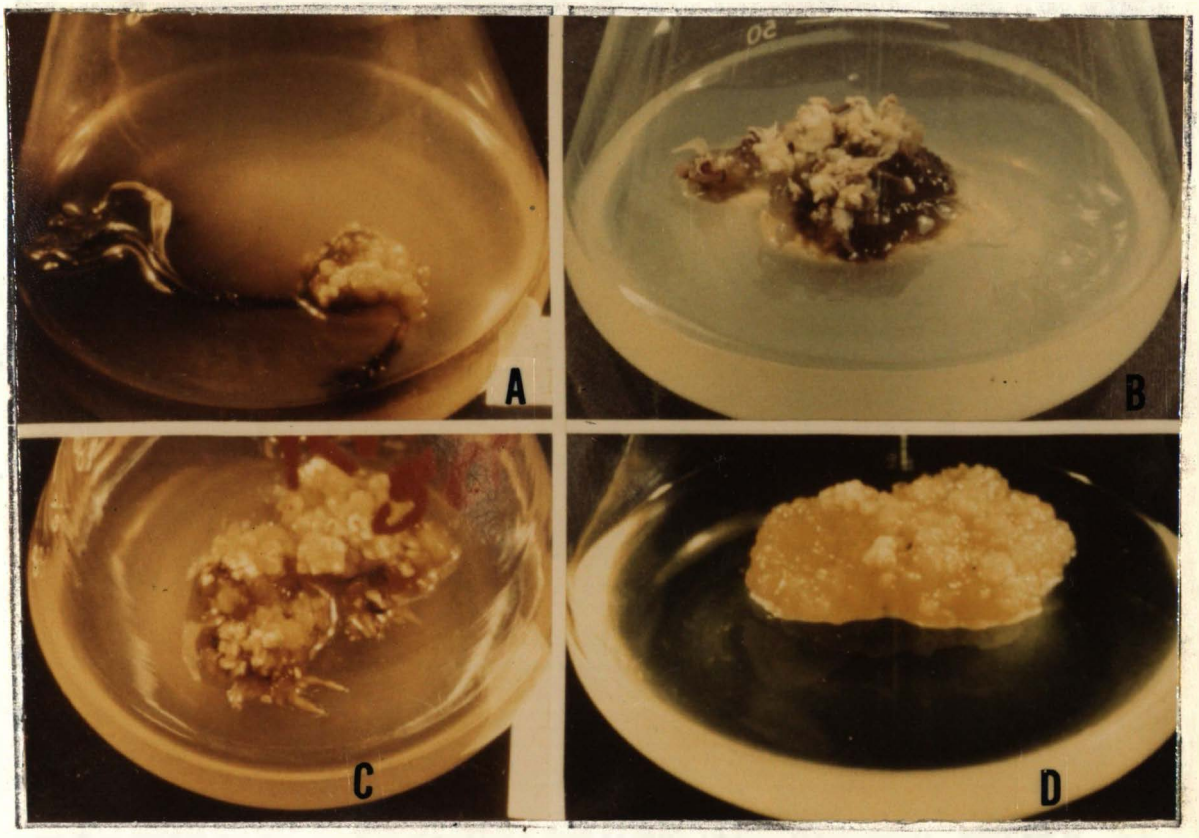


Figure 5. Growth stages of sorghum callus. (A) primary callus at nodal region of young shoot after 2 months in culture, note blackening of leaves and medium (B) secondary callus after removal of parent tissue and passage to fresh medium, note black tissue and small roots (C) secondary callus after several transfers to medium containing 2,4-D and coconut milk (D) true callus tissue with no root formation after 6 months or more in culture.

medium after the removal of parent tissue. After the first subculture, the callus tissue continued to grow and divide as shown in Fig. 5B. After several transfers a callus tissue was produced which was practically devoid of differentiated roots and black tissue, Fig. 5C. Finally a callus of the type shown in Fig. 5D was obtained

but only after 6 or more months of growth and provided that the right growth conditions had been satisfied. All callus growth stages were maintained on a RM62, coconut milk and 2,4-D culture medium. Stages A, B, and C (Fig. 5) were usually easy to produce while D represents a callus growth stage that was hard to obtain. Callus shown in D Fig. 5 was grown for 1½ years in continuous culture. No shoot or bud formation was ever observed at any callus growth stage.

When sorghum callus at growth stage B or C (Fig. 5) was placed in liquid RM62 plus a sugarcane water extract at 10%, sucrose at 2.5%, and a pH of 5.6-5.8, small roots would form on the callus piece within one week (Fig. 6A). Shaking was necessary for rapid root formation (10 rpm Fig. 1). A water extract from sugarcane stalks, coconut milk, low 2,4-D concentrations, or no 2,4-D were tried as supplements in liquid culture. Root formation occurred in all cases but was enhanced with sugarcane extract. No root formation was obtained with primary callus tissue (Fig. 5A).

After 2 or 3 weeks in the same culture medium or after transfer to fresh medium, callus pieces with roots and small root segments eventually turned black (Fig. 6B). Transfer of callus pieces with roots to solid medium of the same composition as liquid did not prevent blackening.

In liquid shake cultures, single cells and clumps of cells were often sloughed off into the culture medium (Fig. 6C,D). At first many single cells would be evident and often adhere to the sides of the culture flask. As soon as organ differentiation began, less cell

sloughing occurred. A mixture of small cells, intermediate sized cells, to giant cells (Fig. 6C,D) was characteristic of liquid shake cultures. Single cell division was not observed in sorghum cells found in liquid culture.

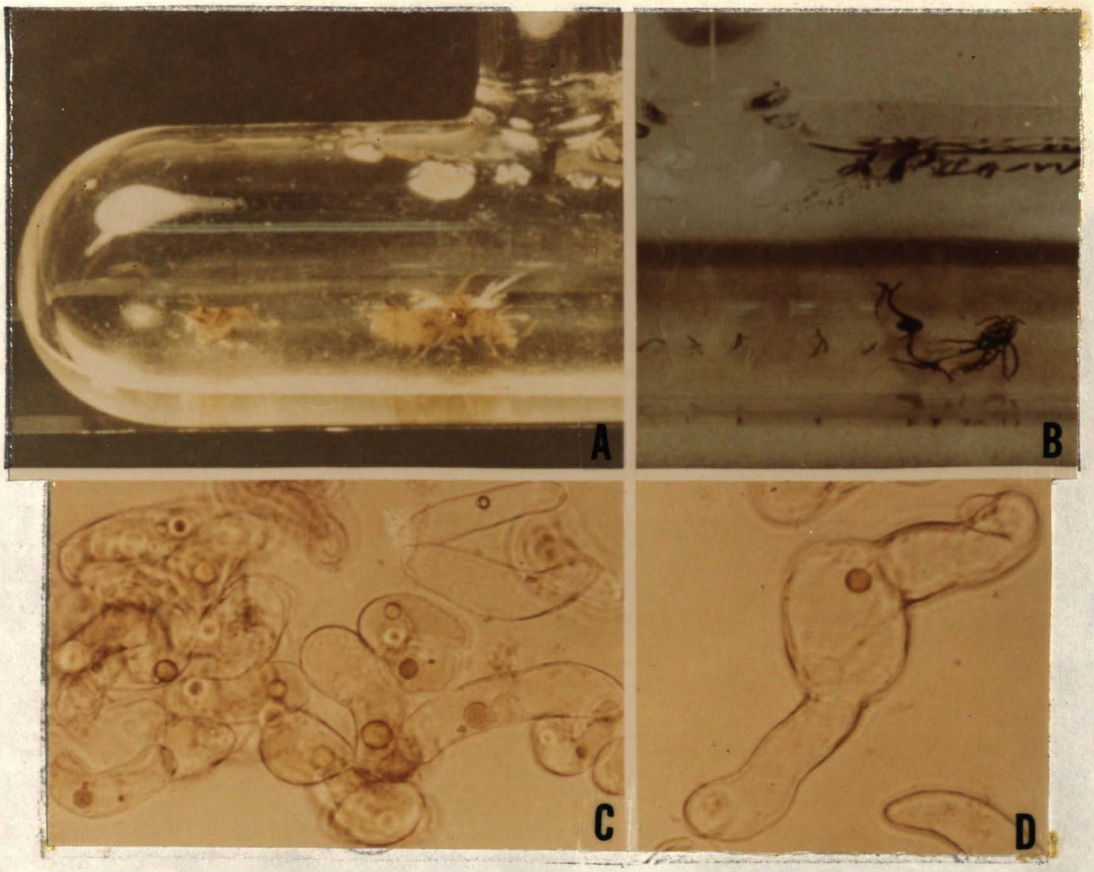


Figure 6. Sorghum callus and single cells grown in liquid culture. (A) sorghum callus in liquid culture, note root formation (B) sorghum callus with roots after several weeks in liquid culture, note blackening (C) single cells produced in liquid shake cultures, note brown bodies (D) giant single cell, note brown body, nucleus, and cytoplasmic strands. Phase contrast photomicrography magnified X500

Some cell division was observed in callus pieces grown on agar medium (Fig. 7A-D). These callus pieces were similar to growth stages C and D (Fig. 5) and were stained with aceto-orcein, squashed, and temporarily mounted. A very low mitotic index was observed and consequently accurate chromosome counts could not be obtained. The cells shown in Fig. 7A and B do appear to be in a polysomatic condition as compared to a normal 2n chromosome complement of 20.

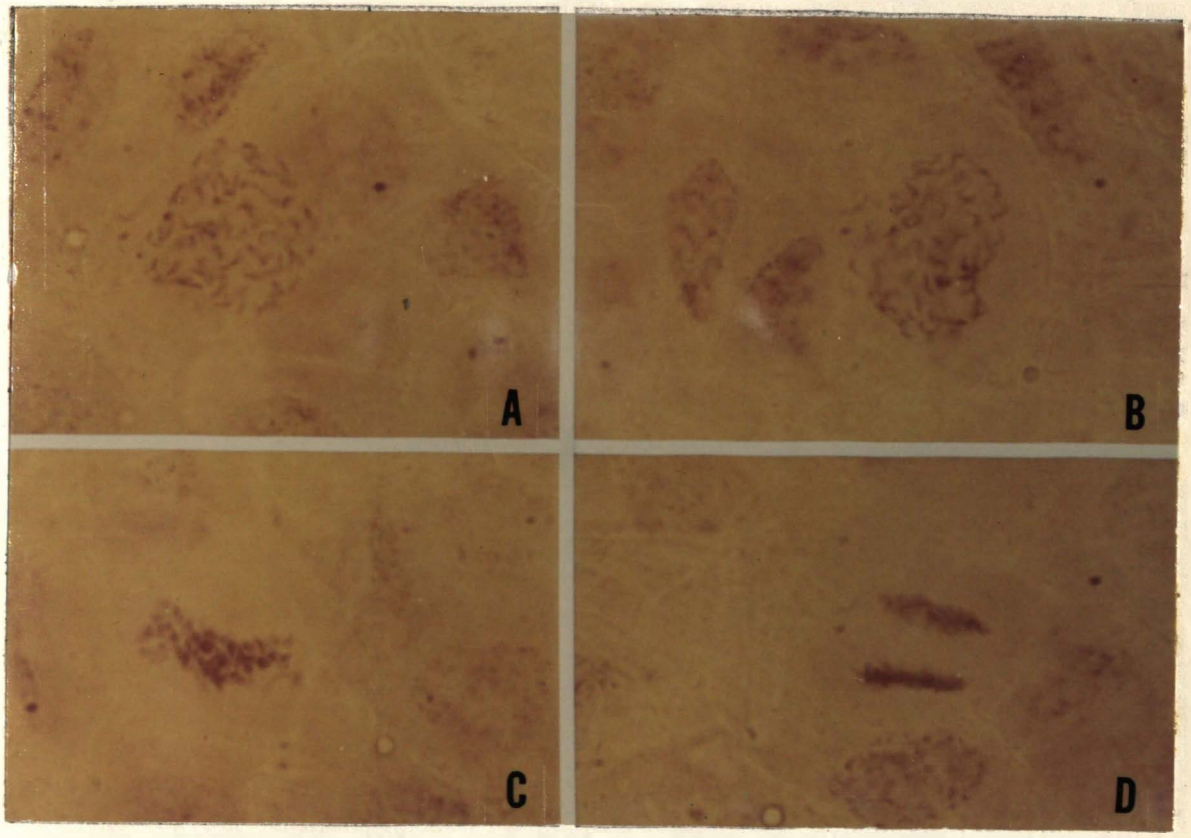


Figure 7. Sorghum cells in mitosis. (A and B) sorghum chromosomes in prophase (C) chromosomes in metaphase (D) chromosomes in anaphase. Magnification X1125

Blackening was a common occurrence in the culture of sorghum callus (Fig. 3, 5, and 6). Within a short time after excision the stump of the internode would turn black, and after several weeks in culture the coleoptile sheath would turn black (Fig. 5A). Blackening occurred most readily on the basal medium used for callus initiation. Blackening of sorghum tissue also resulted in a discoloration of the culture medium which was detrimental to callus growth. A method was sought that would prevent discoloration of the culture medium.

It was suggested that polyvinylpyrollidone (PVP) might prevent blackening. PVP is a chemical compound reported to form stable insoluble complexes with tannins, especially those which form strong H-bond complexes with protein (Loomis and Battaile 1966). Soluble PVP was available in three molecular weights (MW): 10,000, 40,000, and 360,000 and was added to the culture medium at 1% by weight before autoclaving.

In Fig. 8A, sorghum shoots grown for 2 weeks on basal RM62 medium and sugar (4%) were prevented from blackening the agar medium with 10,000 MW PVP added as compared to the control. After 1 month in culture the PVP at 10,000 MW had allowed some blackening to occur as compared to the other PVP MW's tested (Fig. 8B). Sorghum shoots grown on RM62 basal medium plus 2,4-D at 5 ppm and coconut milk (10%) for 1 month showed very little difference at any MW of PVP and little difference from the controls (Fig. 8C). Sorghum callus that was in the process of blackening (Fig. 8D left) and grown on PVP 10,000 MW

medium (Fig. 8D right) discolored the medium after a short time in culture.

Some characteristics of the black pigment produced by sorghum tissue are the following: it is slightly soluble in organic solvents and in acids; it is very soluble in strong alkaline solutions being bleached completely from sorghum leaves by a 1M KOH solution. In connection with the possible synthesis of the black pigment, the amino acid tyrosine was found to be present in young sorghum shoots by means of the Millon's test.

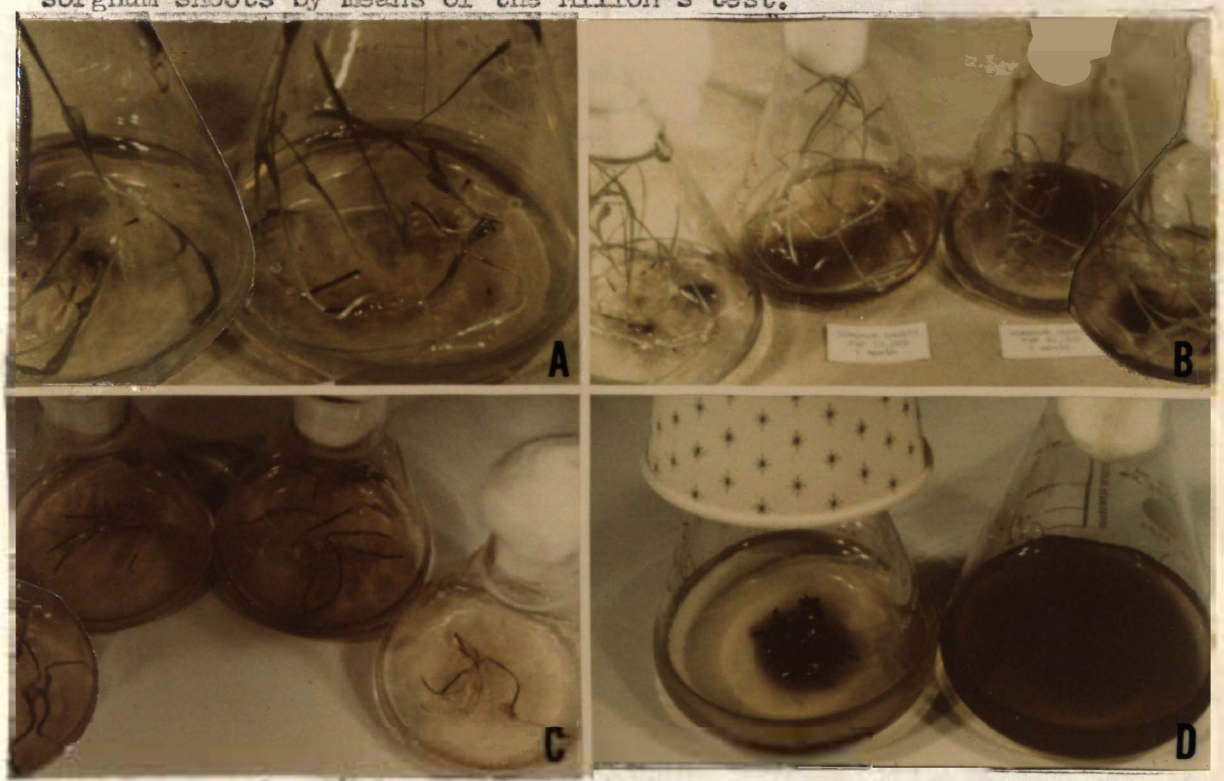


Figure 8. Blackening and suppression of blackening in the culture medium. (A) sorghum shoots grown on control medium (left) and 10,000 MW PVP medium (right) (B) sorghum shoots after 1 month on from left to right control, 10,000, 40,000, and 360,000 MW PVP (C) sorghum shoots grown on callus initiating medium for 1 month from left to right control, 10,000, 40,000, 360,000 MW PVP (D) blackening sorghum callus (left) that was grown on medium containing PVP (right) at 10,000 MW.

Brown bodies were found in single cells (Fig. 6C,D) and in cells of black tissue (Fig. 9). They were also observed in the cells of black lower leaves found on green house grown sorghum plants. The brown bodies were usually under 10 μ in diameter (Fig. 9A) but some as large as 20 μ in diameter were observed (Fig. 9B). There was usually only one brown body per cell. Brown bodies stain red when stained with safranin and fast green.

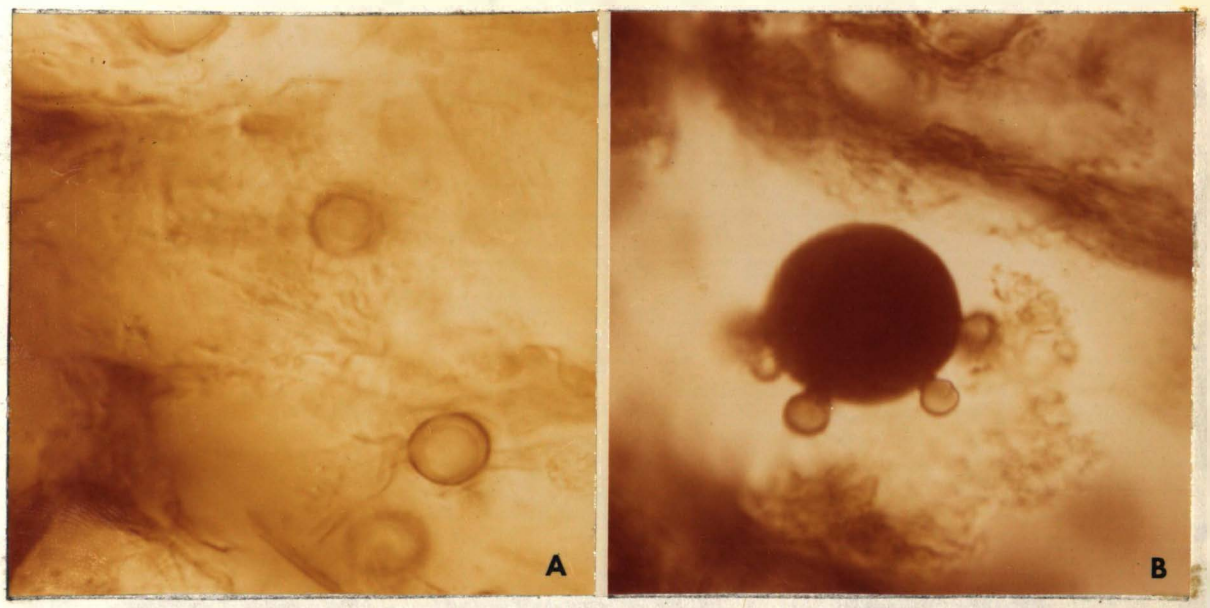


Figure 9. Inclusion bodies associated with black sorghum tissue. (A) brown bodies found in black callus tissue (B) one budding brown body approximately 20 μ in diameter. Magnification X1125

One additional result that should be noted is the effect of IAA on sorghum seedlings variety N.D. 104 and on sorghum callus tissue. When IAA at 10 ppm was present in the culture medium, massive abnormal root formation occurred (Fig. 10B) compared to normal primary and secondary roots in the controls (Fig. 10A). Shoot growth was

decreased when grown on medium containing IAA, but no callus growth was observed after 3 weeks in culture. Callus tissue similar to growth stages B and C (Fig. 5) responded in two different ways to high concentrations of IAA (100 ppm) as shown in Fig. 10C,D. After 3 weeks in culture, the callus shown in Fig. 10C had formed many roots compared to the callus tissue shown in D of Fig. 10 which had not produced roots. No bud or shoot formation was observed in either case.

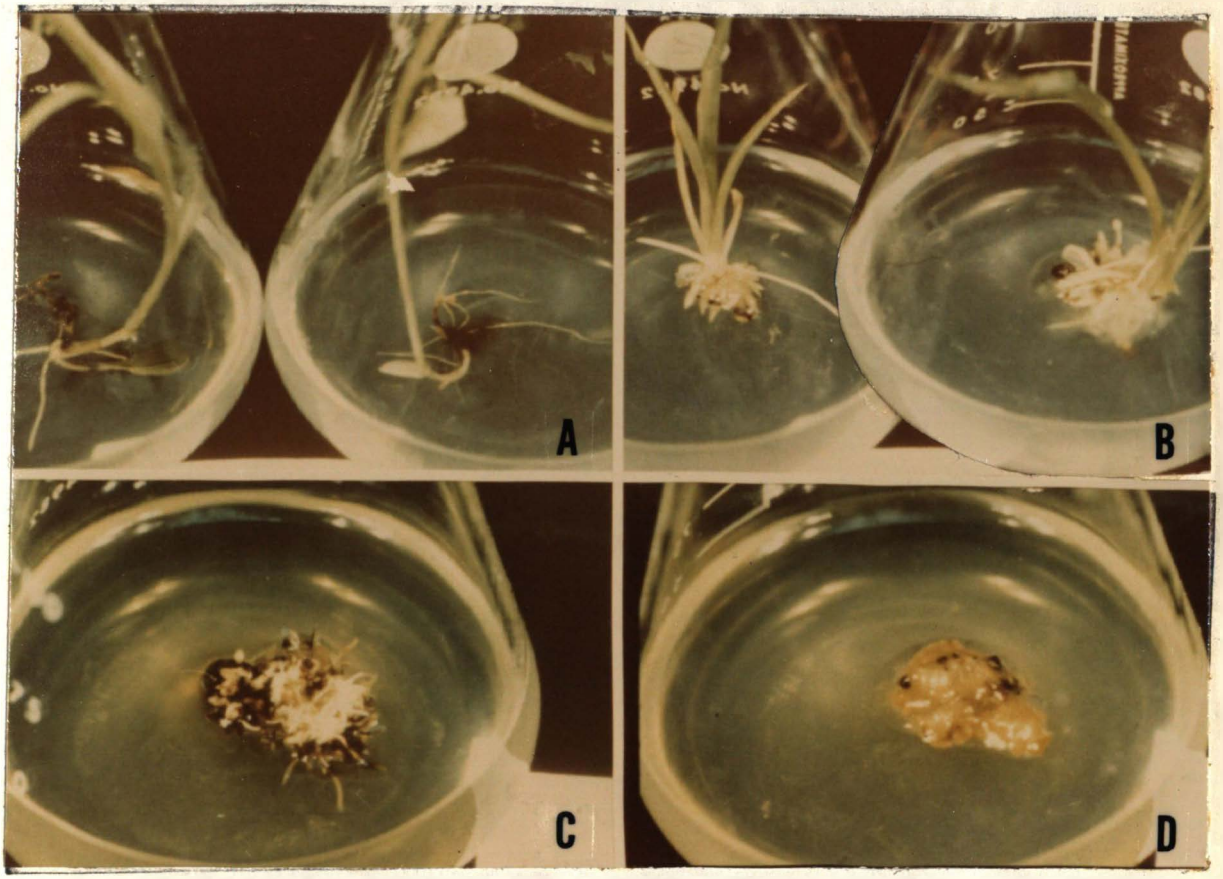


Figure 10. Effect of IAA on sorghum seedlings and sorghum callus tissue. (A) sorghum seedlings grown on control RM62 for 3 weeks (B) sorghum seedlings grown on RM62 + 10 ppm IAA for 3 weeks (C and D) sorghum callus grown on RM62 + 100 ppm IAA and 10% coconut milk for 3 weeks.

DISCUSSION

This study has described the conditions necessary to produce callus from sorghum shoots and the anatomy of callus origin. Attempts to demonstrate cell totipotency following methods used by Sun (1966) with carrot were only partly successful. The problem of blackening and the presence of associated cell inclusion bodies were also investigated.

The data plotted in Fig. 2 indicate that 2,4-D at the concentrations tested does not significantly affect germination but does affect shoot and root length. The highest concentration tested showed pronounced inhibition of root growth but did not completely inhibit shoot growth. The growth response of sorghum roots and shoots to increasing 2,4-D concentration is typical of plant organs as shown in Devlin (1966) p. 410. From the data in Fig. 2, it was possible to choose a range of 2,4-D concentrations that might initiate callus growth from shoot tissue but suppress organized growth (root growth).

An assumption was made that for tissues whose nutrient requirements were unknown, a nutrient medium should be used that is high in salt content. For this reason Murashige and Skoog's (1962) culture medium was used since it is considered high in nitrogen and potassium as compared to other media used in plant tissue culture. The choice of RM62 is also justified by recent reports on monocot callus culture and the use of RM64 as the culture medium (Carter et. al.

1967, Wilmar and Hellendoorn 1968). RM64 (Linsmaier and Skoog 1965) differs from RM62 only in the amount of vitamins; RM64 contains only thiamin while RM62 contains thiamin and others.

Coconut milk was also assumed to be necessary for callus growth although all the factors for its growth promoting activity have not been identified. Experiments not included in this research did show that callus could be obtained without coconut milk but at a much slower rate than when coconut milk was present. Coconut milk may enhance growth by its synergistic action with 2,4-D (in Willmer 1966 pp. 498,605, Steward et. al. 1967).

The important variable then was the amount of growth regulator needed to initiate callus. Fig. 3 showed that callus growth could be obtained in a range from 1 to 7 ppm 2,4-D, since at 10 and 15 ppm callus growth was depressed. From the results obtained in Fig. 2 and Fig. 3, a 2,4-D concentration of 5 ppm was chosen for the initiation and subculture of callus tissue. At this concentration it was found that undifferentiated callus could be produced in a relatively short time (Fig. 5). Similar results were obtained by Webster (1966) with 2,4-D at 5 ppm and oat callus.

The length of time needed to obtain a callus of the type shown in Fig. 5D is very long compared to dicotyledon callus cultures of tobacco (Murashige and Skoog 1962) and carrot (Sun 1966). Callus growth of this type is hard to produce, possibly due to the following reasons: primary callus must be transferred during its exponential period of growth; blackening may stop growth completely; variability

in callus origin; injury of callus; or too small an inoculum size. Callus of this type appears similar to carrot callus which can be grown indefinitely and still display cell totipotency (Sun 1966) but apparently the sorghum callus has lost this ability. The long growing period and difficulty to establish in culture seriously limit the usefulness of sorghum callus for research studies.

Although some root differentiation occurs in callus growing on agar medium with 2,4-D (Fig. 5), a much more vigorous root formation occurs in liquid culture with shaking. Shaking was used to provide aeration and to slough off single cells (Fig. 6) for the possible formation of embryoids. However, only root formation occurred and no shoot or buds were ever observed. This is similar to results obtained by other workers with monocots (Mascarenhas et. al. 1965, Trione et. al. 1968). Wilmar and Hellendoorn (1968) have reported the successful culture of asparagus cells to embryoids, but this condensed report is somewhat lacking in its explanation of embryoid origin and histogenesis. Its reproduceability is questionable especially when compared with the work of Steward et. al. (1967) and Yatazwa et. al. (1967).

Single cells shown in Fig. 6 are similar to those reported in the literature (Steward et. al. 1967, Trione et. al. 1968). These cells have few cytoplasmic strands and show little sign of organization which are possible causes or results of their slow growing nature and inability to divide (Steward et. al. 1967). This slow rate of growth is characteristic of sorghum callus and is seen by

the small number of mitotic divisions found in one callus piece (Fig. 7). These divisions can not be assumed to be representative of the whole callus piece or of other callus growth stages, but they might indicate a tendency to become polysomatic with increasing age of callus (Murashige et. al. 1967). This might account for the apparent loss of totipotency displayed by callus growth stage D-Fig. 5. The production of sorghum polyploids in tissue culture may be of value to the plant breeder since tetraploid tobacco cells have still been able to display totipotency (Murashige et. al. 1967).

Blackening of the culture medium by sorghum tissue can only be delayed for a short time by the addition of PVP to the agar medium. The results shown in Fig. 8 indicate that PVP might prevent blackening for a short time in medium with only sucrose but soon becomes ineffective with time especially when 2,4-D is present. The ability of PVP to prevent blackening would depend on the secretion of phenolic substances by the sorghum tissue. Sorghum tissue might secrete phenolic compounds that become oxidized to pigments but do not form H-bond complexes with PVP. Loomis and Battaile (1966) state that it is probable that some phenolic compounds which do not form strong H-bonded complexes with proteins or PVP are readily oxidized to quinones. These compounds would not be removed effectively by adsorption on PVP. Other possible reasons for the ineffectiveness of PVP are that it may not be readily available in the culture medium and therefore needed in larger amounts or it may be broken down in autoclaving which would seem unlikely.

The black pigment described in the results has all the indications of being melanin pigment. Melanins are naturally occurring nitrogenous polymers formed by the action of oxygen, in the presence of tyrosinase, on tyrosine or closely related compounds (Thomas 1955). Melanin pigment in plants has not been widely studied with the most work being done with animals. The preliminary tests for properties of the pigment from sorghum tissue are not conclusive but are very similar to those described by Thomas (1955) for presumptive melanin pigments in plants. There have also been reports of pigmentation in other monocots in tissue culture such as in corn (Mascarenhas et. al. 1965, Tamaoki and Ullstrup 1958) in wheat (Trione et. al. 1968) and others (Morel and Wetmore 1951). Butenko (1968) also sites references which report of increased polyphenol oxidase (tyrosinase) activity in the presence of both IAA and 2,4-D or with aging of the tissue. He concludes that the brown coloration or blackening is usually linked with melanin formation. Blackening in sorghum shoots must result from the initial wounding by excision of the shoot and the subsequent disruption of enclosing tissues as the callus growth is formed. Blackening of callus tissue growing on solid or liquid medium may result from injury to cells during transfers, from the presence of 2,4-D and coconut milk, or from an unsuitable micro-environment found in shake cultures.

The occurrence of brown bodies (Fig. 6 and 9) in black areas of sorghum callus and scale leaves, and in inactive single cells would suggest a relationship to the black pigment formation. The sectioned

appearance of brown bodies in tissue sectioned with the freezing microtome (compare Fig. 6 with Fig. 9) and the property of staining red with safranin would suggest the presence of a limiting membrane around the brown body. Brown bodies may be the site where melanin formation occurs or is stored, many cells with brown bodies may give the tissue a dark appearance. Similar cell inclusion bodies, arbitrarily called brown bodies in this research, have not been described in the literature to the author's knowledge.

The response of plant tissues to 2,4-D has been reported to be much greater than to the natural auxin IAA (Webster 1966, Yatazwa et. al. 1967). As shown in Fig. 10, IAA at two times the concentration of 2,4-D was unable to initiate callus in sorghum seedlings. Instead, it appears that profuse root development was promoted but without the cell proliferation and fusion that occurs when 2,4-D is present (Fig. 4). Similar results as those of Webster (1966) with oat callus were obtained when a high auxin concentration was tried for callus growth (Fig. 10). The callus tissue that was unable to form roots may have been inhibited by high auxin or have lost the capacity to form roots.

CONCLUSIONS

1. Callus tissue can be produced from the monocotyledon Sorghum vulgare by the use of a basal medium supplemented with coconut milk and 2,4-D.
2. The herbicide 2,4-D at 5 ppm was found to be satisfactory for callus initiation and growth in subculture. Callus could not be produced by replacing 2,4-D with IAA.
3. The presence of 2,4-D in the culture medium inhibits young sorghum shoots and stimulates adventitious root formation and cell proliferation in the nodes of excised shoots. The expanding lateral roots fuse by means of parenchyma cells to form callus tissue.
4. Sorghum callus growth may be divided into three morphological growth stages consisting of primary callus formed on the original explant, secondary callus with differentiated roots on the first subculture, and true callus tissue on subsequent subcultures.
5. A polysomatic condition may be a characteristic of the true callus tissue.
6. Sorghum callus formed roots most readily in liquid culture but no shoots were formed.
7. Single cells are produced in abundance in liquid culture but cells failed to develop into embryos and, therefore, cell totipotency could not be demonstrated.
8. Sorghum tissue grown in solid or liquid culture tends to produce a black pigment. This black pigment is similar to melanin.

Attempts to prevent pigmentation and discoloration of the culture medium were unsuccessful.

9. A cell inclusion body called a brown body was found in single cells, callus tissue, and normal sorghum tissue. Its association with black tissue suggests a relationship to melanin.

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