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The method of radicle emergence in Phytolacca americana (pokeweed)

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THE METHOD OF RADICLE EMERGENCE IN *PHYTOLACCA AMERICANA* (POKEWEED)

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Phytolacca americana

Reproduced from "*Guide to Flowering Plant Families*" Wendy B. Zomlefer (1994)

ABSTRACT

Germination is a key element in the development of a plant. This process encompasses several stages, one of which is the elongation of the radicle tip. This process has been noted to occur by different methods such as rapid mitotic division or cell elongation. However, such a mechanism has not been studied in *Phytolacca americana* (pokeweed)..

It was the intention of this study to observe at the cellular level the nature of the *Phytolacca americana* radicle during normal germination. This was done using a routine histological microtechnique which involves embedding in paraffin and sectioning with a microtome. The sections were then observed under a microscope for cellular activity.

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Acknowledgements

I would like to acknowledge all those who helped make this research possible. I would like to begin by thanking the Biology Department and the Honors program at the University of Tennessee-Chattanooga for making this research possible. I would like to give a special heartfelt thank you to Dr. Barbara Walton for her guidance and knowledge of botanical histological micro techniques. I would also like to thank Becky Bell for ordering the proper supplies needed to complete my research; Dr. Edwards for sharing his journals and knowledge of pokeweed; and Dr. Van Horn for letting me repeatedly walk through his office to get to the pokeweed lab.

Although I could not state the method by which the radicle emerges, I was able to develop a successful method to isolate the small radicle. I also have developed several suggestions, not only as to what is being observed at the cellular level, but methods which can be taken to find out the process of radicle emergence.

INTRODUCTION

Phytolacca americana, also known as pokeweed is a weedy species found among warm regions worldwide. The pokeweed is known for its toxic properties which have important eco-physiological roles that most likely contributes to its success in distribution. This weed has become of interest in many fields of study including: immunology, biochemistry, physiology and ecology. Yet with this new found research, many mechanisms of the pokeweed are not understood.

One of these mechanisms is germination. Germination is the emergence of the root tip, or radicle, from the seed. This process includes the transformation of dormant tissues into active and growing structures that change rapidly. Germination can occur by many different mechanisms such as: elongation of cells, cell division or a simultaneous effort of elongation of cells and cell division (Mayer and Poljakoff-Mayber, 1989). The above types of germination have been observed in many spermatophytes (seed plants), yet this process in the pokeweed has not been identified.

The purpose of this Biology Honors Research project was to observe, at the cellular level, the nature of the radicle during normal germination.

PHYSICAL DESCRIPTION

Phytolacca americana is also known as poke, pokeweed, and American nightshade. It is a hardy perennial herb. Its heigth ranges anywhere from 1-3 meters. This plant can be identified by its many stems coming from the crown having alternate leaves that are glabrous and shaped lanceolate to elliptic-lanceolate (8-30 cm long, 3-12 cm wide). The raceme can vary in length from 5-20 cm and can be erect to nodding,with a bracteate pedicel. The white (sometimes purple) flowers are perfect, having staminate and pistillate parts present on flowers with sepals persistent in fruit. The purplish-black berry is 5-12 carpellate (carpels-ovary chamber). On average, each berry contains 10 seeds. The shiny black seeds are flattened and about 2.5-3 mm long. The plant has a chromosome count of 2n=36. These plants are in flower from May up until the first frost (Radford *et al.*, 1964).

NAME DERIVATION

The species *Phytolacca americana* was originally described by Linnaeus. The name *Phytolacca americana* is composed of several root names (derivations). *Phyto-* comes from the Greek word meaning "vegetable or plant". The term *-lacca* is Latin for "purplish-red pigment prepared from a resinous substance". The word *americana* indicates the origin of the plant (Jones, 1987).

HISTORY

Pokeweed can be found in disturbed areas like gardens and along fence lines. Pokeweed has a majestic appeal to its form due to its reddish smooth stem and deep purple berries. Many people because of its beauty have chosen not to remove the plant once it has penetrated their property, however, they often quickly return to cut it down because of its offensive odor. This is just a mere example of pokeweed's paradox.

This plant has had a long history within this country and Europe. Native Americans of the eastern U.S. would use different parts of this multi-purpose plant. They would eat the very young shoots; make

medicine from its thick, fleshy roots; and used the juice for dye in clothing, crafts, and ink (Ogzewalla *et al.*, 1962). The pokeweed was intentionally exported during colonial times to Europe (Sauer, 1952). In Europe, the French and Portuguese added the juice of the pokeberry to their wine to enhance the color. This wine was perceived as of a higher quality, thus allowing them to increase the price. However, to their dismay, the pokeberry juice spoiled the flavor of the wine so this practice had to be suspended. Since its European arrival, the pokeweed has grown outside its original limits and can now be found widespread throughout Europe (Jones, 1987).

As mentioned before, Native Americans are not the only ones who have partaken of the pokeweed plant as a source of food and medicinal properties. A common practice among many groups today is to boil the young shoots of the pokeweed so that it can be safely eaten. The shoots are generally washed twice after boiling to ensure that all the poisons are removed. The stalk can also be fried like the vegetable okra because the grease destroys the poison in the stalk (Crellin and Phillpot, 1990).

Written records of pokeweed's miraculous cures can be dated as far back as 1817, when George Heywood gave an account in the New England Journal of Medicine which in summary established pokeweed's reputation as being "emetic and cathartic in appropriate doses" (Crellin and Phillpot, 1990). Pokeweed has had a significant role in homeopathy because of such abilities. Cures in the form of capsules and teas could be used for diseases of the respiratory tract, as a laxative, and to decrease pain and inflammation caused by arthritis. It has been further noted to help laryngitis, tonsillitis, mumps, and cases of syphilis (Jones, 1987; Crellin and Phillpot, 1990). It is also believed by many herbalists to cure skin ailments like hemorrhoids, insect bites, and boils. Pokeweed, however, is not only associated with solving ailments of humans, but has been noted to have significant effects on animals. Extracts from dried roots with added lanolin help to treat swollen udders on cows (Jones, 1987).

With such accounts, there is no doubt one can come to understand its benefits. Pokeweed, however, is also a very dangerous and toxic plant. Ingestion of the toxic plant, including seeds, can cause vomiting, convulsions, and possibly death. Ogzewalla (1962) reported

that in Oklahoma during the Fall, warnings would be posted in regards to poisonous plants of the area. These warnings informed the public that children may possibly be tempted to eat the brilliantly purple berries. They warned that if such ingestion occurred serious results would ensue like CNS depression, convulsions, and possibly death from respiratory arrest. With such reports, Ogzewalla became interested in finding the source of toxicity of pokeweed. In his study, he found that the roots were the most toxic portion of the plant and they contained alkaloids. The toxicity of berries was assumed to be caused by the same alkaloid component. It is not absolutely certain, however, that the toxicity is based on this single compound alone. Although we know pokeweed is toxic, we are unaware of its fatal dosage (Ogzewalla *et al.*, 1962). In spite of the lack of knowledge, according to expert wild food gatherers, the shoots should never be collected when they have a "tinge of red" and one should never include any root when cutting the stem (Jones, 1987).

NATURAL HISTORY

Pokeweed's origin begins in the New World with a wide range mainly North of the Tropics. This vast area includes almost the entire eastern half of the United States up through southeastern Canada and even including remote areas in northeastern Mexico. Although it is stated that pokeweed covers the eastern half of the United States, there are gaps throughout this range. Such gaps can occur at mountainous regions, which suggests that pokeweed's northern border is dependent on temperature. This temperature dependent relationship was noted by Sauer (1952) who observed that pokeweed was not found where the temperature average was below 20 ^{o}F. He further cited, that the summer temperatures are more critical than winter temperatures. Sauer concluded that the northern boundary is based on duration of temperature above the minimum requirement for proper plant growth; while the western border is determined by moisture rather than temperature (Sauer, 1952).

With pokeweed's wide distribution, we cannot lose sight of the fact that it is found principally in disturbed areas. The key to the idea of disturbed areas suggests pokeweed's downfall-- competition.

Pokeweed does not have the capability to thrive in an area that has been established by plants that are highly selected to that locality. When pokeweed has been well established after several seasons, it can live a few years with competition but, in the end, pokeweed will be selected against (Sauer, 1952).

However, pokeweed's inability to compete with other plants has not slowed its distribution. Pokeweed's success has been based on its ability to tolerate temperature, light, and moisture conditions. Yet the ultimate contributor to its wide range, is the 29 different bird species that consume its berries (Armesto *et al.* 1983). McDonnell (1984) headed a study in which it was found that birds actually chose the type of raceme upon which to feed. The raceme is picked on the basis of several factors: amount of ripened fruit, higher mean fruit weight, pulp weight, and pulp seed ratio. In this study, McDonnell concluded that an average raceme holds up to 78 ripe fruits with an average of 10 seeds per fruit. Another factor that enhances seed consumption by birds is pokeweed's ability to have berries ripening all the time. Armesto (1983) reported that of 381 seeds removed from bird droppings, 84% germinated after 8 days. When the factors above are

combined with the presence of two major peaks of dispersal, one in late October and one in Mid-November, the distribution of pokeweed is enhanced. McDonnell concluded that with the long ripening period, attractive berries, and high levels of toxin present, these combined factors helped to ensure that birds are the main dispersors. The combined factors, in turn, deter mammals (McDonnell *et al.*, 1984).

RELATIVES

Phytolacca americana is the predominant species of its family in the eastern United States. Recently, there has been discussion on the proper taxon for *P. rigida*, Small. Many taxonomists believe that P. rigida is a separate species and not *P. americana* because it has erect raceme versus *P. americana*'s drooping raceme, and that its location is restricted to Florida and the Gulf and Atlantic coasts. However, Caulkins and Wyatt (1990) disagree with the idea of *P. rigida* being a separate species. They artificially cross-pollinated the two individuals and ended up with viable seeds. They further studied the biochemical components of each plant and found that their flavonoid compositions were indistinguishable. They found that in each plant studied, seed germination and tolerance of salinity showed no difference. They did,

however, after analysis of 14 morphological characteristics in 36 populations, find that *P. rigida* did have several distinct and different characteristics from *P. americana*, such as pedicel length,raceme length, and berry width to name a few. Even with the above results, they concluded that *P. rigida* was not distinct enough to be called a separate species, but was rather a variety of *P. americana* (*P. americana* var. *rigida*) (Caulkins and Wyatt, 1990).

AUTOTOXICITY

Recently, here at UTC, pokeweed's autotoxicity has been researched by many students including Kevin Anderson (1995). In his honors project last year, he tried to isolate the inhibition agent in pokeweed juice. In 1988, Dr. Edwards and several students published a paper in the American Journal of Botany in which they examined what parts of the pokeweed plant caused intraspecific inhibition or autotoxicity. In their study, they found that pokeweed seeds would not germinate in the presence of pokeberry juice concentrations $\geq$ 20% v/v. They found that the pokeberry juice maintained its inhibitory capabilities even after juice was autoclaved. They further concluded that mature leaf and root extracts ($\geq$50%) caused complete inhibition,

while immature leaf extracts did not. These studies suggested that different tissues contain varying amounts of the inhibitory factor (Edwards *et al.*, 1988).

It has been suggested that seeds that are inhibited may have an advantage over those that are not. This type of behavior would be beneficial in preventing autumnal germination. These inhibitors are suggested to be found in newly matured seeds giving protection from premature germination in combination with temperature dependency and also a hard testa. (Edwards *et all.*, 1988). Sauer (1952) has suggested that disturbed areas could stimulate germination.

REPRODUCTIVE BEHAVIOR

Phytolacca americana is a polycarpic herb that has flowers that set fruit at a high percentage. It has been found that the number of fruits are dependent on the size of the raceme while the number of racemes increases with increased plant height. On average, the berry contains about 10 seeds. Armesto (1983) claims that unstratified seeds germinate at about 80%. Yet this figure varies with area, climate, and period when berries were collected. In this study, Armesto found that germination of seeds varied between 25% and 99% in the 25 different

individuals that were collected versus seeds collected from one individual plant with variance of between 3 and 86%. From the information given, this suggests that pokeweed seeds from different berries on the same raceme have variable germination probabilities. This is most likely due to varying degrees of maturation because of pokeweed's indeterminate nature of its raceme. Armesto *et al* found that the percentage of germination in seeds was not related to weight of fruit, but, instead, was based on the physiological conditions of the berry. In this study, Armesto and his colleagues collected a sample in New Jersey and found that, on average, there are 58 berries per raceme and that one shoot can have up to 754 berries (average=154). However, only a certain percentage of these berries became ripe at any particular time (Armesto *et al.*, 1983).

As noted above, on average, each berry contains 10 seeds. However, as with any average, there are always berries containing higher and lower quantities of seeds. Variations from the average seem to be related to ovule abortion. The cause of ovule abortion is believed to be the result of poor pollination and, hence, poor fertilization caused by fungal-infected pollen grains. According to

Mikesell (1988), who conducted the study near the Maryland-Pennsylvania border, she found that anywhere from 40% to 100% of ovules she examined aborted. In her study, she concluded that ovules are aborted during "the formation of the female gametophyte or the globular embryo." Evidence indicating that ovule abortion has taken place is when you have more ovules than seeds in the fruit (Mikesell, 1988).

DORMANCY

This is the stage of development prior to germination. The seed itself is in a suspended state. The method by which dormancy is ceased varies among species. Dormancy can be caused by factors such as chemical and/or mechanical. These dormancy inducing factors can be seen in the Pokeweed. For example, a mechanical factor is the hard testa which does not allow penetration of oxygen and water needed to induce germination. Dormancy can be ceased by many methods such as acid scarification or cracking the seed coat. However, in nature, this process can occur by many means: passage through the digestive tract of an animal, climatic changes (e.g., a pre-chilling requirement), or running water over seeds like a river bank

(Salisbury and Ross, 1992).

The second form of dormancy is one of chemical means. The word chemical implies inhibitors which are present that do not allow germination to take place. This method can also be seen in pokeweed as described previously. It is believed that the water-extractable inhibitor is in highest concentration in berries, roots, and mature leaves (Edwards *et al.*, 1988).

To further exemplify the mechanical dormancy, the matured prickly sida (*Sida spinosa*, L.) seeds are water impermeable like the pokeweed seed. They will remain so until the seed coat becomes permeable to water. Through much study, it has been determined that arrest of dormancy could be lost at the chalaze area and this could possibly be the same for the pokeweed (Egley *et al.*, 1986).

GERMINATION

According to Graeme P. Berlyn (1972) in the book titled Seed Biology, germination can be described as, "the sequential series of morphogenetic events that result in the transformation of an embryo into a seedling." This dormant period can range from less than one week in the sugar maple (*Acer saccharinum, L.*) to 90 years in the

clover (*Trifolium striatum, L.*). Pokeweed seeds have been found to be viable for quite a lengthy time. In one particular case, seeds that were buried in 1902 and found in 1941 had an 80%-90% germination rate (Sauer, 1952).

Storage of seeds can affect viability. It has been shown that seeds stored under moist conditions usually will have a decreased viability while some, however, remain unaffected (*Juncus* species). The best conditions to store seeds to maintain their viability is in a area with no water and low temperatures. If the seeds are exposed to high temperatures and low water the seeds will dehydrate and rupture upon rehydration.

There are four stages to germination (Salisbury and Ross, 1992):

1.) Imbibition. This is the first process by which water is introduced (hydration). The water penetrates the embryo and hydrates proteins and other colloids.

2.) Formation and/ or Activation of Enzymes. When the enzymes are functioning they increase the metabolic activity of the embryo.

3.) Elongation of the Radicle. This stage is signified by the emergence of the radicle from the seed coat.

4.) Subsequent Growth of the Seedling. This stage is marked by characteristic features associated with underground growth till time of emergence from soil.

Imbibition

Shull (1916) discovered this mechanism in cocklebur seeds when he monitored the uptake of water after having noticed an increase in weight. He noticed an initial increase in weight, followed by a plateau, and then a second gain in weight. The first increase was the stage where the seed tissue was hydrated (Leopold and Kriedemann, 1964).

It has been found that imbibition is reversible during the initial increase in weight. Six hour imbibed embryos have the ability to be repeatedly dehydrated and rehydrated without causing inviability. However, once growth of roots and meristems occurs, germination cannot be reversed by dehydration. Such dehydration at this point in development will result in cell death (Bradbeer, 1988).

Formation and/or Activation of Enzymes

Shull (1916) noticed that the plateau stage marks the point where the seed establishes the needed metabolic system for growth. The enzymes needed for metabolic activity can be derived from two places (Leopold and Kriedemann, 1964):

1.) released or activated from existing proteins or;

2.) made *de novo* by way of nucleic acid directed protein synthesis.

Elongation of the Radicle Tip

As Shull (1916) further described, the second weight increase signifies radicle emergence indicating a greater rate of oxygen utilization (Leopold and Kriedemann, 1964). As previously stated, this is the stage whereby emergence of the radicle from the seed coat occurs. In the book, International Series of Monographs on Pure and Applied Biology (Mayer and Mayber, 1963), the question is asked whether elongation of the radicle caused by the process of cell elongation or cell division. This question was answered by Haber and Luippold (1960) who used gamma radiation to determine by what means the radicle elongates. In their study, they found that cell

elongation was the cause of radicle protrusion. However, both cell elongation and cell division have been noted in a number of species even one occurring after the other (Mayer and Mayber, 1963).

Subsequent Growth of the Seedling

In the final stage of germination, once the radicle emerges, the plant remains subterranean until the epicotyl reaches the surface of the soil. While underground, the plant receives nourishment through the cotyledons (Leopold and Kriedemann, 1964).

STATEMENT OF PROBLEM

Within the past two decades, the importance of pokeweed is becoming more significant. This heightened interest stems from its importance in the field of medicine as a possible means of cancer treatment, not to mention, its presumed allelopathic remedies seen in its treatment for arthritis. In spite of such marked interest in the pokeweed, little is known about the plant's mechanism of growth, and in particular its method of seed germination, whether it be cell division or cell elongation. In my Biology Honors Research project, I propose to observe, at the cellular level, the nature of the radicle during normal

germinaton. This process shall be done with the use of a histological microtechnique which involves embedding in paraffin, sectioning the specimen with a microtome, followed by a staining process using Safranin-Fast Green and Iron-Alum Hematoxylin as two separate staining agents to allow for observation at the cellular level.

MATERIALS AND METHODS

COLLECTION AND PROCESSING

The pokeberries were collected during late October of 1995. These pokeberries were collected off of Hamill Road in Hamilton County, TN along some railroad tracks. Entire racemes were cut from the plant; each raceme was chosen based on the appearance of its berries (purplish-black indicating ripeness). The sample of berries was collected from approximately 30 plants that were widely distributed, in order to ensure genetic variability. Whole racemes were collected and stored in paper bags and placed in a refrigerator. The racemes were removed from the refrigerator and were placed on racks and washed with tap water. The racemes were then allowed to air dry for a day. The berries were then separated from their raceme and placed in two

sieves (one on top of the other). While running tap water on the berries, they were pressed against the sieve with the fingertips to separate the seeds from the berry. The seeds would pass through the first sieve (4mm x 4mm mesh) and be collected by the bottom sieve (2mm x 2mm mesh). The seeds were rinsed with distilled water until seeds were clean from remaining pulp or juice. To further clean the seeds and distinguish between the seeds that were mature versus those that were not (floaters), the seeds were placed in a 1000 ml beaker with distilled water. Those seeds that remained submerged were kept. The mature seeds were then collected and allowed to dry on paper towels. Once the seeds were dry, they were placed in a glass container sealed with parafilm and stored at 4 ^{o}C.

INDUCTION OF GERMINATION

Seeds were induced to germinate by cracking their hard outer coating (testa) with a pair of pliers. Each seed was held between the thumb and index finger while pressure was applied along the circumference of the seed mainly in the area around the funiculus (See Fig. I.). Pressure was sustained until cracking occurred. Once the seeds were cracked, they were placed in a small petri dish lined by

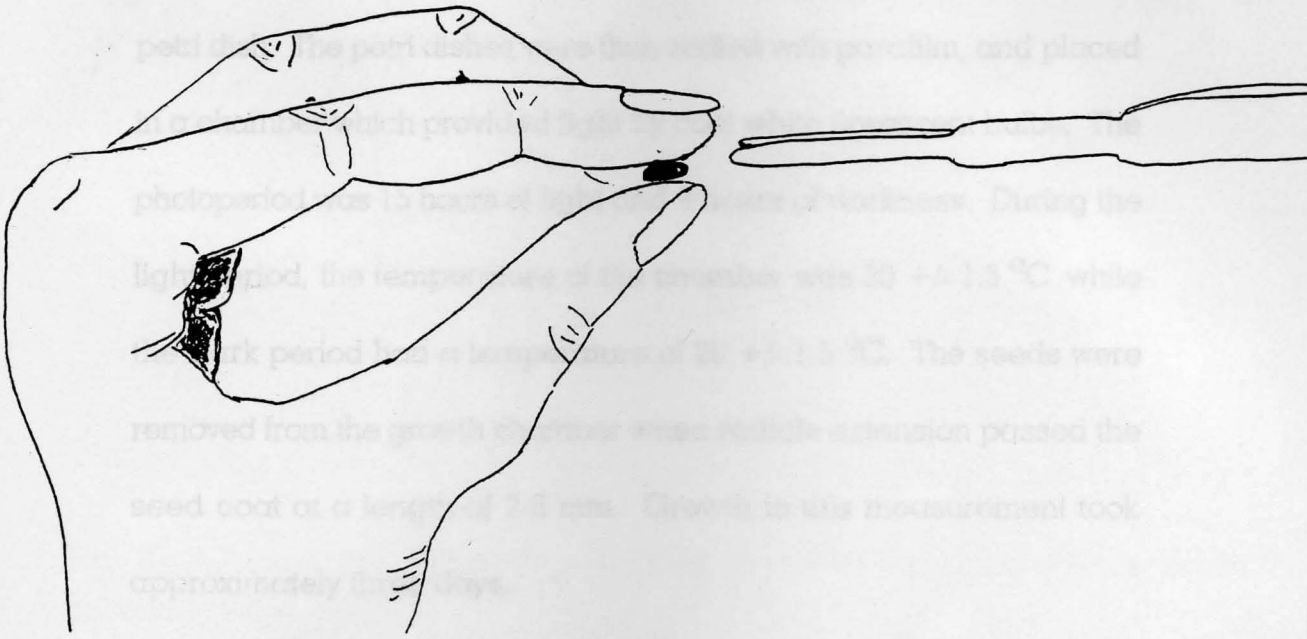


Fig. I. Diagram showing proper alignment for cracking the pokeweed seed.

filter paper. Approximately 10 ml of distilled water was added to each petri dish. The petri dishes were then sealed with parafilm, and placed in a chamber which provided light by cool white florescent bulbs. The photoperiod was 15 hours of light and 9 hours of darkness. During the light period, the temperature of the chamber was 30 +/- 1.5 ^{o}C ,while the dark period had a temperature of 20 +/- 1.5 ^{o}C. The seeds were removed from the growth chamber when radicle extension passed the seed coat at a length of 2-3 mm. Growth to this measurement took approximately three days.

Once germination was achieved, a seed was placed within the groove of a folded piece of masking tape (See Fig. II., A.). Next a razor was placed between the hard testa and the embryo along the crack of the seed. The hard testa was removed by pulling the razor toward the body while using the thumb as a form of resistance and pushing in the opposite direction (See Fig. II.,B.). The tape was turned around and the hard testa was removed from the other side of the embryo. The radicle end of the embryo was then cut away with a razor from the remainder of the embryo and testa, resulting in isolation of the radicle tip along with additional embryonic tissue (See Fig. III.). With the use of

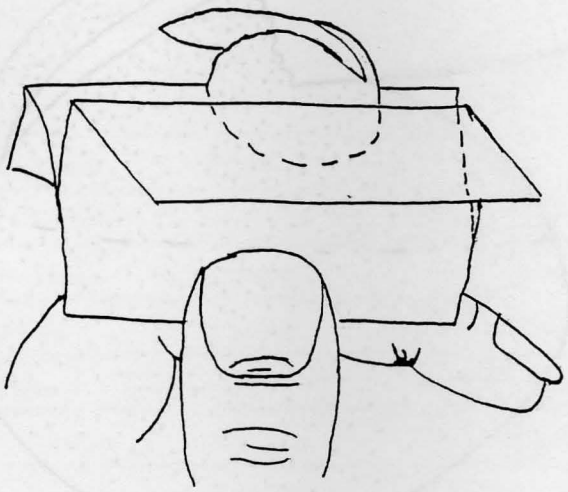


Fig. II., A. Diagram indicating the placement of the pokeweed seed in the masking tape.

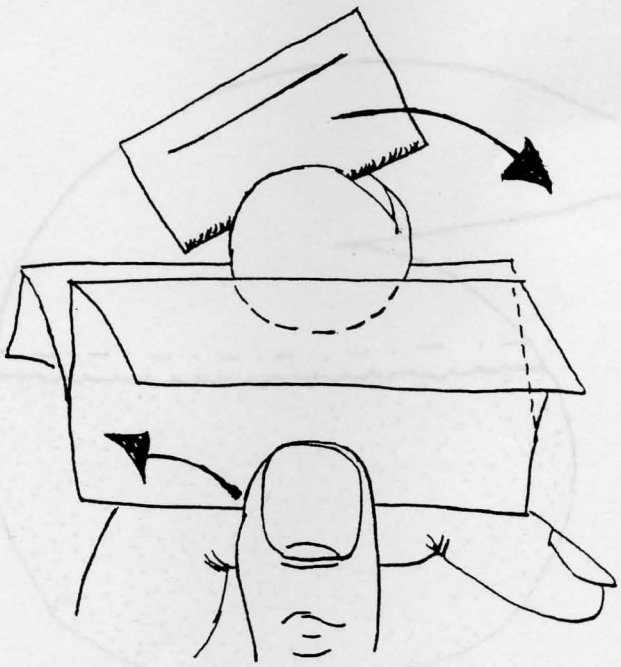


Fig. II., B. Razor method which allows for removal of hard testa from pokeweed seed.

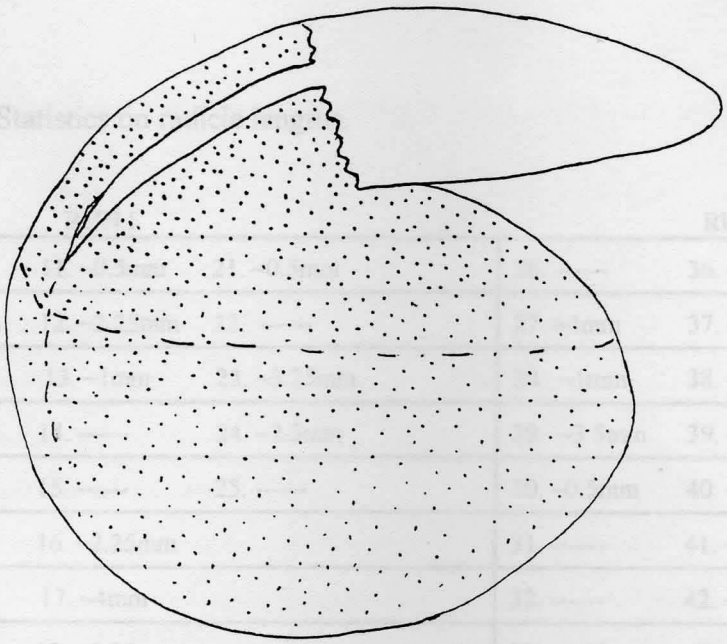


Fig. III., A. Drawing which indicates proper removal of hard testa (indicated by dashed lines).

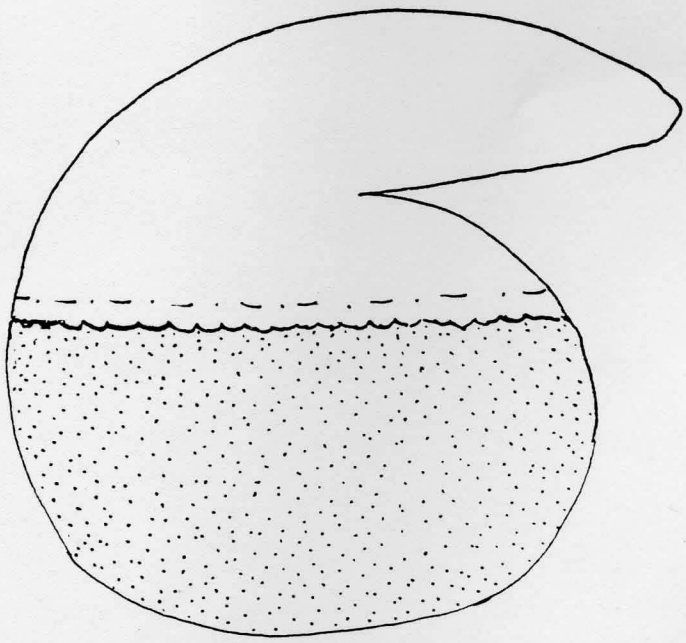


Fig. III., B. Drawing which indicates proper portion of radicle and embryo removed for processing (indicated by dashed lines).

Table 1. Statistics on radicle lengths.

RUN I.			RUN II.		
1. ~1mm	11. ~0.5mm	21. ~0.5mm	26. ------	36. ~4.25mm	46. 3.25mm
2. ~0.5mm	12. ~2.25mm	22. ------	27. ~4mm	37. ~1.5mm	47. ~1mm
3. ------	13. ~1mm	23. ~3.25mm	28. ~1mm	38. ------	48. ~1.5mm
4. ~1.5mm	14. ------	24. ~2.5mm	29. ~3.5mm	39. ~2.5mm	49. ~1mm
5. ~4mm	15. ------	25. ------	30. ~0.5mm	40. ~3mm	50. ~3.5mm
6. ~3.5mm	16. ~2.25mm		31. ------	41. ~5mm	
7. ------	17. ~4mm		32. ------	42. ~3mm	
8. ~2.25mm	18. ~2.25mm		33. ~2.25mm	43. ------	
9. ------	19. ~0.5mm		34. ~3mm	44. ~3.25mm	
10. ~2mm	20. ~3mm		35. ~2mm	45. ~2mm	

Embryos chosen for preparation are as designated: E1=4, E2=8, E3=16, E4=18, E5=24, E6=34, E7=35, E8=40, E9=42, E10=48.

Table II. General information concerning radicles used in this experiment.

	RUN I.	RUN II.
# of seeds cracked	25	25
# of seeds that germinated	18	20
% of germination	72%	80%
Range of radicle length	0.5mm-4mm	0.5mm-5mm
Average radicle length for total sample	~2.05mm	~2.55mm
Period of germination	3 days	4 days
# of seeds in the ideal range for experiment (1.5mm-3mm)	8	9
Average length of selected radicle	~2.2mm	~2.5mm

Of those seeds in the ideal range, 5 were chosen from each run for histological preparation. Embryos from Run I are designated E1 through E5 with lengths given in above chart. Embryos from Run II are designated E6 through E10 with lengths given in above chart.

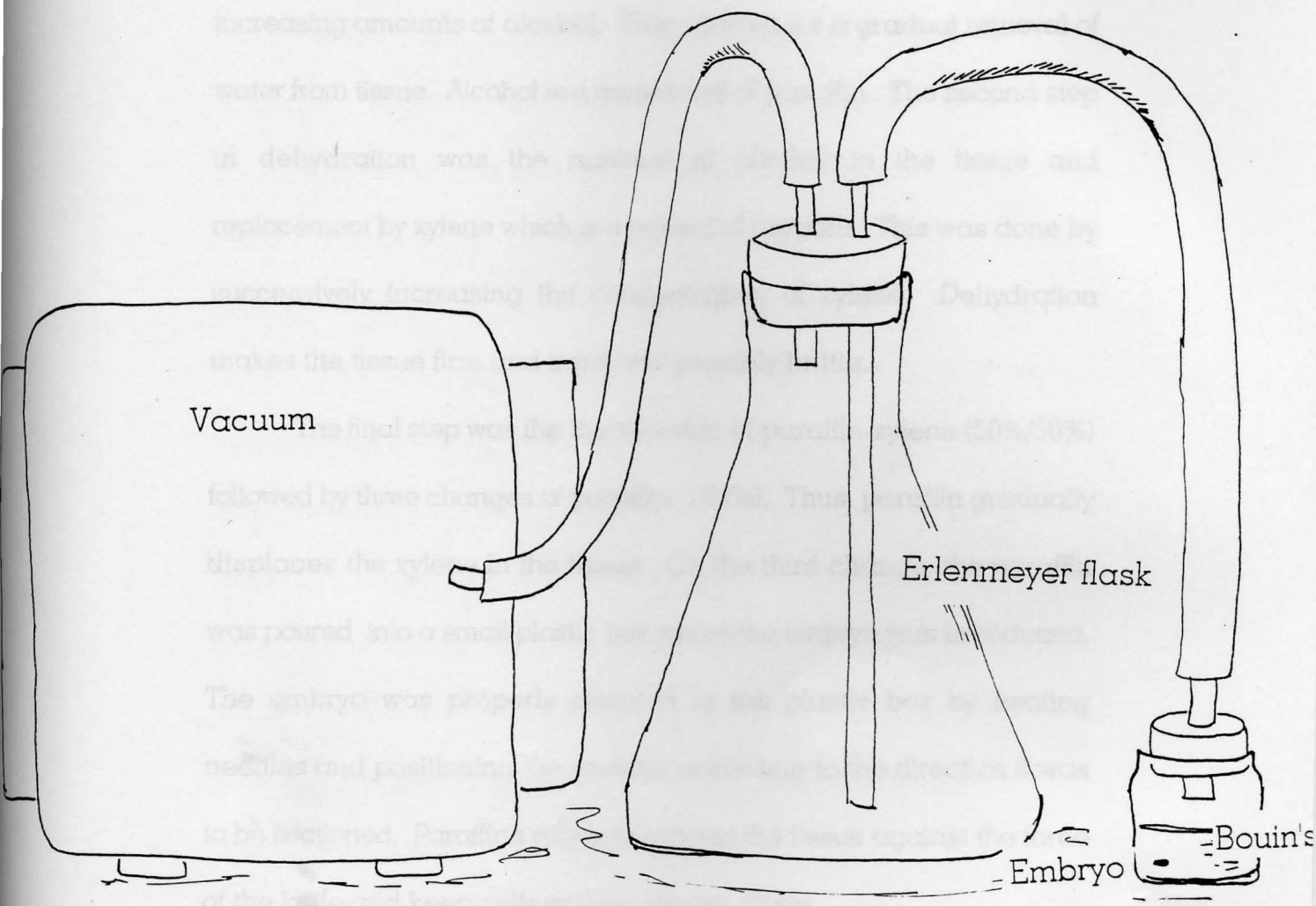
tweezers, the tip was placed in Bouin's solution immediately following excision. In Botanical Microtechnique, Sass (1963) describes the routine procedure of fixation, embedding, sectioning, and staining of plant tissue.

FIXATION OF EMBRYO

It is important for the specimen to be entirely immersed in the Bouin's solution so that complete fixation of the tissue may occur. Because the radicle tip is so small it usually will not sink in the Bouin's solution. In order for it to sink, a vacuum was applied to the vial that contained Bouin's and the radicle (See Fig. IV.). The vacuum pulls the air out from between the cells and allows penetration of the fixative. The application of the vacuum is only necessary once for each embryo.

Fixing is done to stop life processes in the tissue. It kills the protoplasm. It also helps to keep the structures of the cell undisturbed and undistorted. The fixatives used are a balanced combination of solutions which help to minimize shrinking and swelling caused by the other ingredients (checks and balances). The fixative that was used was Bouin's based on the formula: 5% glacial acetic acid, 25% formaldehyde, and 75% picric acid saturated aqueous. Following

Fig. IV. Vacuum apparatus used to make radicle sink in fixative.



fixation the embryo was washed with 20% alcohol.

The next step was to replace any water in the tissue by adding increasing amounts of alcohol. This allowed for a gradual removal of water from tissue. Alcohol is a nonsolvent of paraffin. The second step in dehydration was the removal of alcohol in the tissue and replacement by xylene which is a solvent of paraffin. This was done by successively increasing the concentration of xylene. Dehydration makes the tissue firm and hard and possibly brittle.

The final step was the introduction of paraffin-xylene (50%/50%) followed by three changes of paraffin (100%). Thus, paraffin gradually displaces the xylene in the tissue. On the third change, the paraffin was poured into a small plastic box where the embryo was introduced. The embryo was properly oriented in the plastic box by heating needles and positioning the embryo according to the direction it was to be sectioned. Paraffin's role is to support the tissue against the force of the knife and keep cells in their proper place.

SECTIONING OF THE EMBRYO

The solidified paraffin block with the embedded embryo was removed from the plastic box by peeling away the sides of the container. The paraffin block with the embedded embryo was then trimmed to the proper size for sectioning and attached to a wooden block. The embryo was secured on the block by adding paraffin around the periphery of the paraffin block. The wooden block was placed in the clamp of the microtome. The embryo was then sectioned at a thickness of 10 micrometers using the microtome. Most of the embryos were cross-sectioned with only one embryo being sectioned sagittally. After they were sectioned, the embryos were placed in order in a small cardboard box until slide preparation. During slide preparation the sections were placed on a warmed, lightly-albumined slide that had been covered with water. After approximately one day of drying, the slides were properly stained.

STAINING OF THE EMBRYO

The principle of staining is based on the idea that certain structures have an affinity for certain dyes. There were two different stains used in this research project, in order to find the best possible

stain for the sections. The first was Safranin-Fast Green (See Flow Chart I.). The first stain used was Safranin which is usually applied to the point of overstaining. Overstaining is followed by a process known as destaining whereby the excess stain may be removed. The removal of excess Safranin was accomplished through subsequent washes of 30%, 50%, 70%, and 95% alcohol. The destaining technique allows for an optimal staining contrast in the slides. This procedure of overstaining followed by destaining is a routine practice among botanists. Fast Green is removed from the unlignified tissues more rapidly than from the lignin, cutin, and chromatin. The correct staining contrast is achieved when lignin, chromatin, and sometimes cutin are a bright red, chloroplasts are pink to red, and cellulose walls and cytoplasm are green.

The second stain used was Iron-Alum Hematoxylin also known as Heidenhain's. This stain is primarily a nuclear stain and thus is a good stain for the study of mitosis. The procedure for Iron-Alum Hematoxylin can be seen in Flow Chart II. The specimen was washed first with the mordant (4% Iron-Alum) and then was transferred to a wash of the hematoxylin stain after five changes of distilled water.

FLOW CHART I. (Sass, 1958)

I. INDUCE GERMINATION ========> II. CUT EMBRYO ==========>

III. FIX EMBRYO WITH BOUIN'S SOLUTION
- Apply vacuum till embryo sinks
- Leave embryo in vial of Bouin's for ~24 hours
- Rinse embryo with 20% alcohol

IV. DEHYDRATION

Place in vial of:

1. 50% alcohol for 1hour.
** 2. 70% alcohol for 1 hour.
3. 85% alcohol for 1 hour.
4. 90% alcohol for 1 hour.
5. 100% alcohol for 1 hour with 3 changes.

V. REPLACEMENT OF ALCOHOL IN TISSUE WITH XYLENE

Place in vial of *:

	Alcohol	Xylene
1.	75%	25%
2.	50%	50%
3.	25%	75%
4.	0%	100%

* each for one hour

VI. REPLACEMENT OF XYLENE WITH PARAFFIN

Place in vial of:

** 1. Paraffin-Xylene => 50/50 - 1 hour (on warming table)
2. Paraffin => 3 changes - 1.5 hours (oven)

VII. EMBEDDING

** 1. On third change, add pure paraffin in plastic box at room.
2. Place embryo in plastic box ofter paraffin's addition.

VIII. SECTIONING

1. Sectioning by microtome at a thickness of 10 micrometers.
2. Ribbons are placed in cardboard box and stored at room temperature.

IX. STAINING

1. Jar 1 - 5 min. in xylene.
2. Jar 2 - 2-3 min. in xylene.
3. Jar 3 - 3-6 min. in absolute alcohol.
4. Jars 4-8 - 2-5 min. in 95%, 70%, 50%, 35% and distilled water
5. Jar 9 - 1% aq. Safranin for 10 min.
6. Jar 10 - Rinse slides with distilled water till clear.
7. Jars 11-14 - in 35%, 50%, 70%, and 95% alcohol for 2 min. each time.
8. Jar 15 - Stain in 1% Fast Green for 30 sec.
9. Jar 16 - 3 changes of absolute alcohol for 2 min each time.
10. Jar 17 - 3 changes of xylene for 2 min. each time.
11. Cover section with 1-2 drops of mounting medium (Permount).

** Stopping points

FLOW CHART II. (Sass, 1958)

I. INDUCE GERMINATION =======> II. CUT EMBRYO ==========>
III. FIX EMBRYO WITH BOUIN'S SOLUTION
- Apply vacuum till embryo sinks
- Leave embryo in vial of Bouin's for ~24 hours
- Rinse embryo with 20% alcohol

IV. DEHYDRATION

Place in vial of:

1. 50% alcohol for 1hour.
** 2. 70% alcohol for 1 hour.
3. 85% alcohol for 1 hour.
4. 90% alcohol for 1 hour.
5. 100% alcohol for 1 hour with 3 changes.

V. REPLACEMENT OF ALCOHOL IN TISSUE WITH XYLENE

Place in vial of *:

	Alcohol	Xylene
1.	75%	25%
2.	50%	50%
3.	25%	75%
4.	0%	100%

* each for one hour

VI. REPLACEMENT OF XYLENE WITH PARAFFIN

Place in vial of:

** 1. Paraffin-Xylene => 50/50 - 1 hour (on warming table)
2. Paraffin => 3 changes - 1.5 hours (oven)

VII. EMBEDDING

** 1. On third change, add pure paraffin in plastic box at room.
2. Place embryo in plastic box ofter paraffin's addition.

VIII. SECTIONING

1. Sectioning by microtome at a thickness of 10 micrometers..
2. Ribbons are placed in cardboard box and stored at room temperature.

IX. STAINING

1. Jar 1 - 5 min. in xylene.
2. Jar 2 - 2-3 min. in xylene.
3. Jar 3 - 3-6 min. in absolute alcohol.
4. Jars 4-8 - 2-5 min. in 95%, 70%, 50%, 35% and distilled water
5. Jar 9 - 4% iron alum for 10 min.
6. Jar 10 - Rinse slides with 5 one min. changes of distilled water.
7. Jar 11- 10 min. in Hematoxylin
8. Jar 12- 3 changes of distilled water
9. Jar 13 - Apprx. one minute in destaining reagent.
10. Jar 14 - 3 changes of distilled water followed by 5 min. of running tap water.
11. Jars 15-18 - in 35%, 50%, 70%, and 95% alcohol for 2 min. each time.
12. Jar 19 - 3 changes of absolute alcohol for 2 min. each time.
13. Jar 20 - 3 changes of xylene for 2 min. each time.
14. Cover section with 1-2 drops of mounting medium.

** Stopping points

Since the specimen was stained black, a destaining reagent was used. The destaining reagent removes stain rapidly from the cytoplasm, less rapidly from plastids, and slowly from chromatin. Correct staining contrast occurs when the chromosomes, chromatin in resting nuclei, and middle lamella appear blue-black, while the cell walls and cytoplasm appear colorless. The Iron-Alum Hematoxylin proved to be the better of the two stains for the pokeweed sections.

After each staining process, a few drops of Permount were added to the slide. This was followed by the application of a cover slip to the prepared slide.

RESULTS

After careful study of the prepared pokeweed slides several observations were noted. First, at the root tip, there were prophase stages just inside the outer epidermis along the lateral sides of the root tip (see A and B in Fig. VI.). These cells were believed to be in prophase due to the "ink blot" appearance of their condensed chromosomes. The presence of cells in interphase was observed beginning with the third layer from the outer epidermis. Interphase was common in parenchyma cells and within cells of the stele as well (see C and D in Fig. VI.). Also noted was the lack of mitotic figures at the apical tip of the root cap (see G in Fig VI.).

Another major observation was the process of elongation that was occurring throughout the radicle. As can be seen in Fig. VI., when comparing region C to E there was a notable difference in the length of the parenchyma cells from one area to another. This elongation could also be seen in the stele where the more apical cells were less elongated than those more distal cells of the stele (see Fig. VI., D versus F). In Fig. VII., this overall elongation of the radicle cells can be seen by the gradation from region K to region L.

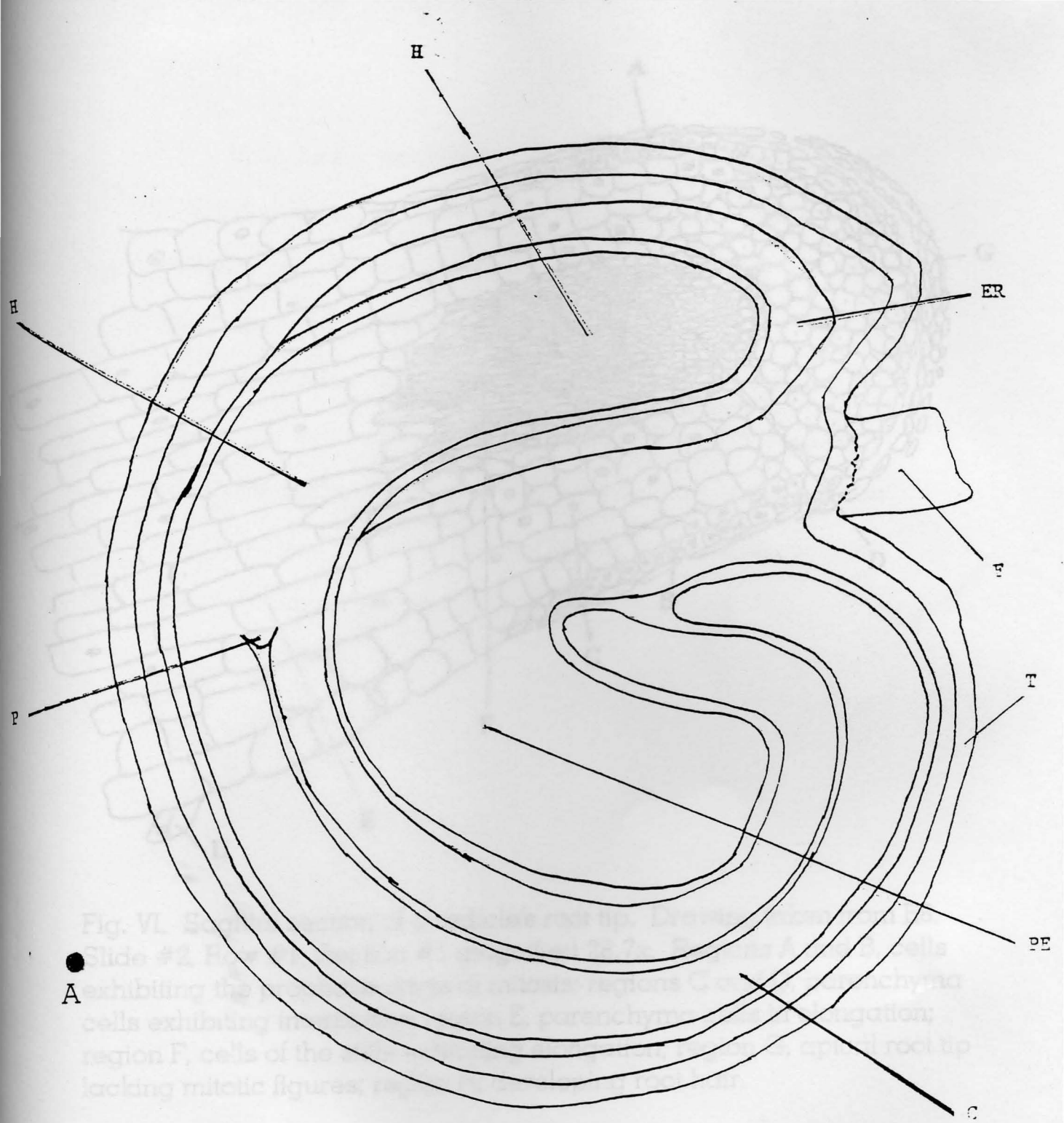


Fig. V. Sagittal section of mature pokeweed seed. The following abbreviations are used in the drawing: ER, cap of crushed endosperm; H, hypocotyl; P, plumule; C, cotyledon; PE, perisperm; T, testa; F, funiculus; A, actual size of pokeweed seed. Redrawn from Woodcock (1925).

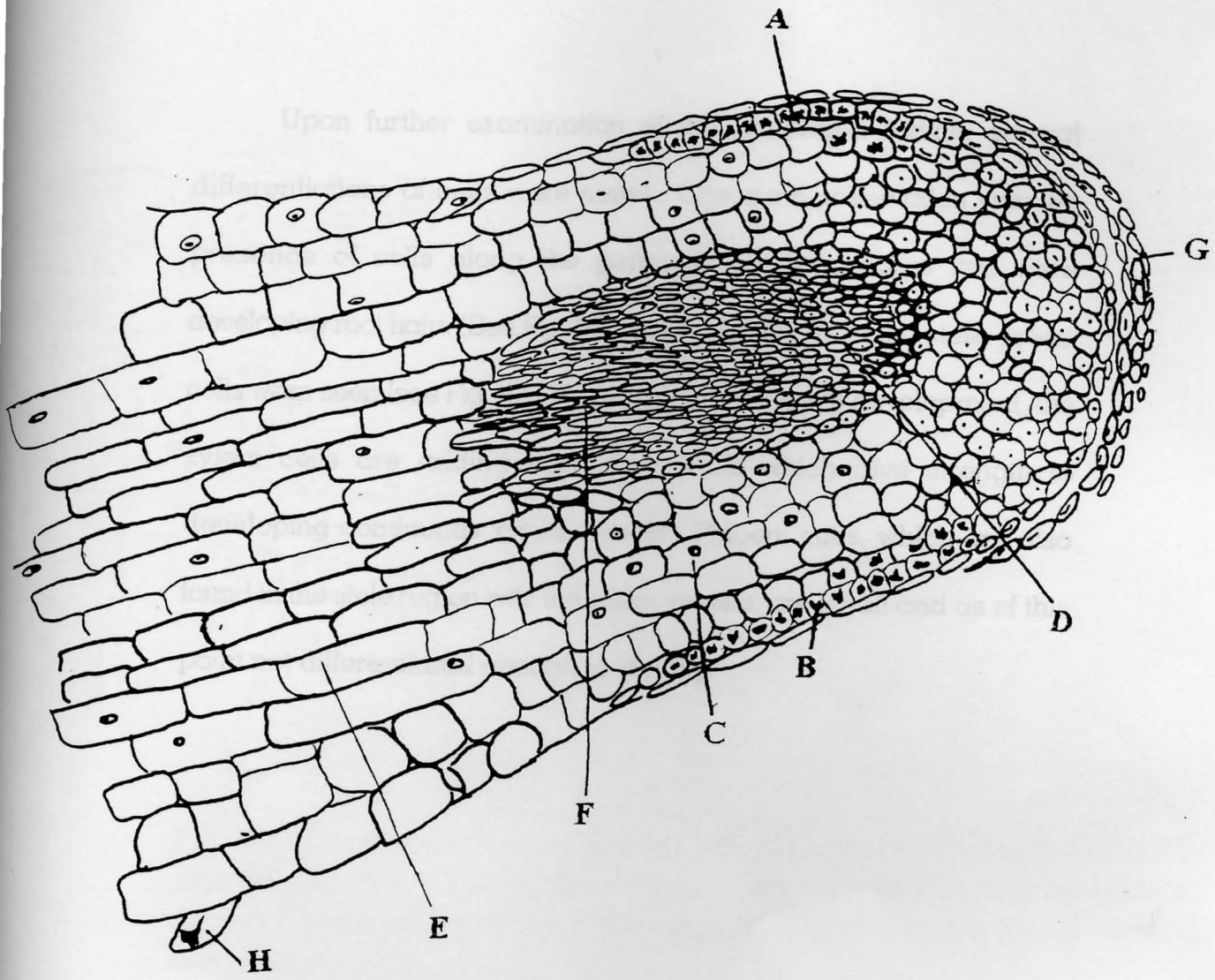


Fig. VI. Sagittal section of a radicle's root tip. Drawing taken from E8, Slide #2, Row #1, Section #1 magnified 28.7x. Regions A and B, cells exhibiting the prophase stage of mitosis; regions C and D, parenchyma cells exhibiting interphase; region E, parenchyma cells in elongation; region F, cells of the stele exhibiting elongation; region G, apical root tip lacking mitotic figures; region H, developing root hair.

Upon further examination of the pokeweed radicle several differentiations of cells were noted. One such observation was the presence of cells along the perimeter of the embryo that were developing root hairs (See Fig. VI., H and Fig. VII.,I). In the stele, xylem cells were seen (see Fig. VII., J.). At this point in plant development, the xylem cells are adding secondary walls which give strength to developing continuous xylem vessels. Phloem cells, which are also found in the stele region with the xylem vessels, are small and as of this point not differentiated enough to identify.

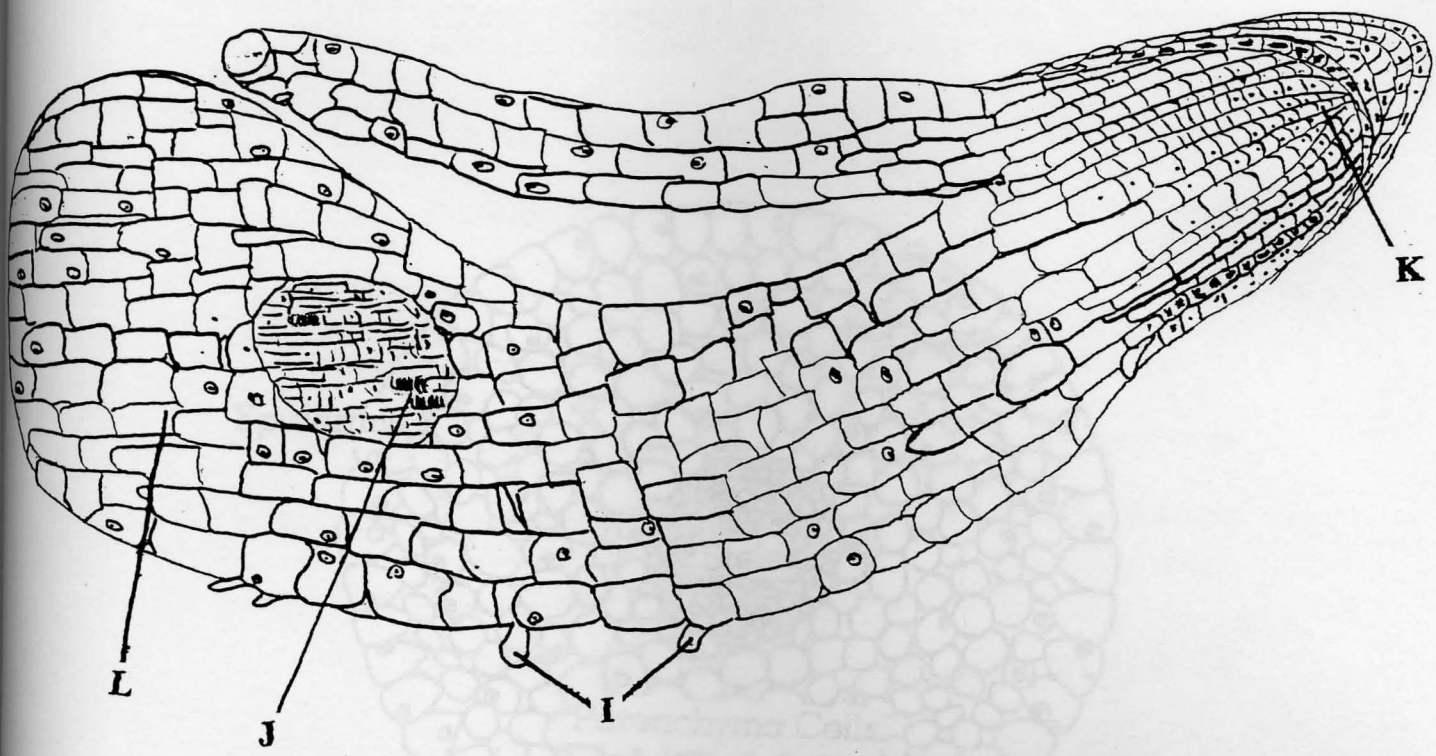


Fig. VII. Sagittal section of radicle. Drawing taken from E8, Slide #3. Row#1, Section #2 magnified 37.6x. Region I, developing root hairs; region J, xylem cells of the stele; regions K and L, radicle cells exhibiting elongation.

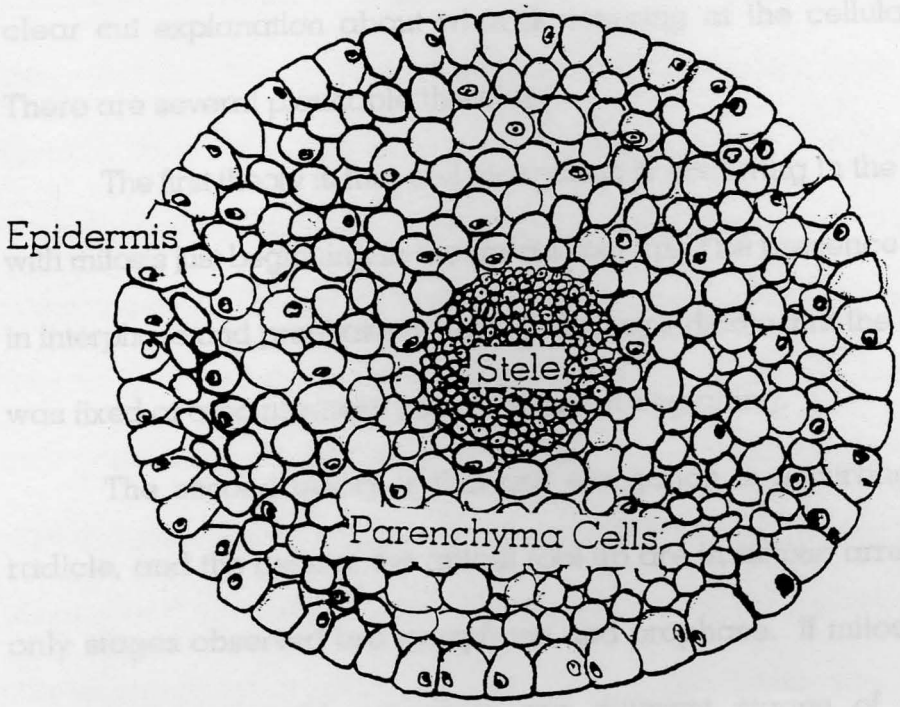


Fig. VIII. Cross section of radicle magnified 61.9x.

DISCUSSION OF RESULTS

In spite of these observations in the pokeweed, there is not a clear cut explanation about what is occuring at the cellular level. There are several plausable theories.

The first theory is that cell elongation is occurring in the radicle with mitosis just beginning in the apical root tip. The presence of cells in interphase and prophase at the root tip may indicate that the embryo was fixed at a point where mitosis was just beginning.

The second theory is that cell elongation is occurring in the radicle, and the cells at the apical root tip are in mitotic arrest. The only stages observed are interphase and prophase. If mitosis were occurring, we would expect to see different stages of mitosis. Therefore, possibly the radicles were fixed at a point of mitotic arrest at least at the radicle tip. The idea that radicle emergence is the result of cell elongation was first explored by Haber and Luippold (1960). It was understood that growth of the embryo occurred by cell expansion and mitotic division. However, it was unknown which came first. Haber and Luippold devised a procedure to study the method by which lettuce seeds germinate using gamma radiation. This study began at the pre-

germination stage in order to observe the cells prior to, during, and after germination. The application of gamma radiation to plant tissue destroys the nuclei of cells so that mitosis cannot occur. They proposed that if radicle emergence were dependent upon mitotic division, then the lettuce seeds would not germinate. However, if the lettuce seeds did germinate then radicle emergence was based upon cell elongation. They found that lettuce seeds germinated. In addition, the root tip upon cellular examination had no indication that there were cells undergoing any mitotic activity at the time of fixation. This further provides evidence that cell elongation and mitotic division are not only processes that occur at different times, but that cell elongation is the mechanism by which the lettuce seed germinates. This second theory may be what is occurring in the pokeweed. However, it cannot be fully explored until sequential fixation of embryos occurs beginning from pre-germination and going through emergence of the radicle.

A third theory is that the tissue of the radicle is not undergoing cell elongation, but rather rehydration of pre-existing cells. Mitotic arrest is at the apical root tip. This is a possibility since cell walls are thinner around the funiculus, thus facilitating easier water absorption

which would be aided through the cracking technique. If the apparent extension of the cells is caused by rehydration, then the cells may not be as elongated in nature because of the artificial cracking. The apical root tip exhibits what appears to be mitotic figures in arrest seen in regions A and B of Fig. VI. The pokeweed could have a diurnal cycle of cell division which has been seen in onion and daffodil, thus explaining the mitotic arrest (Perry, 1996).

A fourth theory is that the elongation of the cells is again the action of rehydration. Cells at the apical root tip covered by a thin root cap are just beginning mitotic division at the time of fixation. This would correlate with the interphase and mitotic prophase stages that were observed. Although this rehydration theory of pre-existing cells may be a possibility, I feel this is not the process that is being observed. These seeds were in the presence of water for approximately three days which is a suitable amount of time for all of the cells within the radicle to be rehydrated. Therefore, the cells at the apical root tip should be more extended than what appears in my slides. For that reason, I strongly believe that the cells in regions E and F of Fig. VI are undergoing cell elongation with the cells at the apical root tip either in

mitotic arrest or undergoing mitosis at the time of fixation. However, such speculation can only be supported through sequential fixation of embryos beginning at the pre-germination stage and following through to radicle emergence.

A final theory is that mitosis has occurred prior to the time of fixation. If this is the case, then the extended cells witnessed within regions E and F of Fig. VI may be due to rehydration or actual cell elongation. The cells viewed at the apical root tip in regions A and B of Fig. VI may be cells which have previously undergone mitosis but have not been elongated either by rehydration or cell elongation. Again, the only possible way to discern the events that have occurred is through sequential fixation of the embryos beginning prior to germination and carried out through radicle emergence.

SUGGESTIONS

I hope that an individual will continue this research using my groundwork as a base. Through the course of my work, I have developed several techniques that will help in the continuation of this research. I begin by advising the use of a vacuum in order to remove air trapped within the embryo. The vacuum allows the embryo to remain submerged throughout processing. The embryo must remain submerged to ensure that all of the tissue has been processed. It is also important that the embryo be embedded so that when it is sectioned both cross and sagittal sections can be obtained. I have also learned that more sagittal sections should be obtained rather than the cross sections in this project. More activity can be seen at the cellular level throughout the entire radicle tip with sagittal sections than with cross sections. Once the embryo has been sectioned and placed on a slide, it should be stained using Iron-Alum Hematoxylin as the preferred staining agent. This stain is extremely helpful in viewing the nuclear portions of the cell.

I would also encourage the practice of sequential fixation of the embryo beginning at the pre-germination stage and following through

past radicle emergence. This would allow one to discern whether mitosis or cell elongation came first. One could further determine if cell elongation is the result of rehydration or a change in microtubule and/or microfilament structure by studying the effect of colchicine (a known microtubule inhibitor) or cytochalasin (a known microfilament inhibitor) on the cells of the radicle.

As for future research on the pokeweed radicle, I would suggest using a sequential collection method starting possibly prior to imbibition and continuing at specific time intervals throughout several days after emergence of the radicle. Using this method would ensure examination of the possible methods by which the radicle of the pokeweed germinates. One could see if there is noticeable elongation of cells due to water uptake accompanied by mitotic arrest. If a diurnal cycle does exist, a sequential method of collecting and processing radicles could lead to the discovery of the onset of mitotic activity. Observations could also reveal whether the radicle undergoes mitotic division at the root tip followed by elongation of more matured cells at the proximal portion of the embryo. With the improved method of isolating the radicle tip, sectioning of the embryo could occur using a

freezing microtome. The ability to isolate the radicle tip from the remainder of the embryo and the hard perisperm could also lead to possible histochemical techniques.

It may seem that there are possibly more questions raised following this research project than before it started. However, great strides have been made in the technique of isolating the pokeweed radicle. Due to the pokeweed seed's size, it was often difficult to remove the hard testa and isolate just the small portion of the radicle. With the improvement of the technique, many more studies can now be done.

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