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DEFENCE MECHANISM IN *CAMPYLOPUS PILIFER* BRID. AND *PORELLA NAVICULARIS* (LEHM. AND LINDENB.)LINDB.: SOME OBSERVATIONS

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Bryophytes, the earliest group of land plants, presents a challenge to our understanding of the process of evolutionary diversification in structure, metabolism and reproduction. Earlier studies of bryophytes have been confined to their biogeography and ecophysiological aspects. From the biochemical stand point, bryophytes accumulate a variety of secondary metabolites, like phenols, glycoalkaloids and phytoalexins as protectives against adverse effects of light, mechanical bruising by predators like beetles, insects and fungi. In the present study, a preliminary attempt was made to trace the analytical and compositional aspects of phenols and their role in host parasite relationships. The two species selected in the present study are *Campylopus pilifer* Brid. and *Porella navicularis* (Lehm. and Lindenb.) Lindb. showed a significant level in the content of soluble phenols and individual phenolics. The major phenolic acids were analyzed and quantified using reverse phase high performance liquid chromatography and their possible role in host defense mechanism is discussed. The relevant physiological effect of poly phenols is considered to be that of astringency based on their ability to complex with proteins. This may render tissue unpalatable to predators and impede their invasion of host tissue. Moreover, high polyphenol oxidase activity in the species was an added boon to the defense mechanism. The activities of major enzymes of phenylpropanoid pathway such as phenylalanine ammonia lyase (PAL) and peroxidase (POX) showed significant activity. The high profile of PAL activity can be correlated with cinnamic acid formation, the precursor of all secondary metabolites. A negative correlation was observed between POX activity and lignin content. The assay data corroborates with histochemical observations. In order to ascertain the role of POX it was fractionated into cell wall bound and cytosolic forms. The rise in the level of cytosolic POX was more unique probably involved in quenching the reactive oxygen species formed in the thallus due to oxidative stress. Thus overall, bryophytes have efficient defense mechanism against phytopathogens or any invaders as well as abiotic stress.

Keywords : Oxidative stress; Phenylalanine ammonia lyase; Phenylpropanoid pathway; Polyphenol oxidase; Reactive oxygen species.

Introduction

Secondary plant metabolites have been an important and growing area of research in recent years[1,2]. An infinitely rich natural products, principally of microbial and plant origin, such as alkaloids, terpines, polyenes, polyacetylenes, phenols and mycotoxins occur sporadically in nature; their presence indeed often constitutes something of a taxonomical speciality. Moreover, they appear to have no explicit function in the economy of the producing organism. The function of secondary metabolism has gained increasing prominence on plant animal co-evolution. Secondary metabolites are produced as a response to environmental and ecological challenge, a chemical armory appropriate to the environmental pressures which they face. Recent works convey that they are produced early in their evolution of plants a characteristic which made them unpalatable by the population of herbivores as defense agents in the plants for survival[3]. This focus is attributed to the expanding awareness and need to prevail upon biological controls to plant pests. Phenolics, their biosynthetic enzymes and their oxidases have been attributed a role in the defense mechanism of many plants. A high turnover of phenolics possibly mediated by the general triggering of the pathways of aromatic biosynthesis or due to enzymic fission of host glycosides by β glycosidases has been

reported in various host pathogen interactions. Further, phenolics and their oxidation products are known for their fungi toxicity[1]. Phenylalanine ammonia lyase (PAL) is the key enzyme for the synthesis of phenolics, phytoalexins and lignin, the three key factors responsible for resistance. Hence PAL has been considered as the most important enzyme in inducing disease resistance in plants. Plant peroxidase (POX) consist of multiple isoenzymes, which play an integral role in the induction of systemic resistance, as a defense mechanism of plants, against pathogens in addition to the last steps in the oxidative coupling of phenolic monomers in the formation of wall polymers[4]. Bryophytes are leafy thalloid amphibian plants and lack complex tissue organization, yet they show diversity in form and ecology. This ecologically significant group was paid little attention and not even properly documented. Another notable feature of the group is that they are relatively free from pathogenic attacks. This resistance may be due to their natural anti-microbial or immunological properties. In view of these facts, an attempt has been made to study the biochemistry of host response to defense with reference to phenols and allied enzymes.

Materials and Methods

Plant Materials - The whole study was focused on two taxa of bryophytes viz. *Campylopus pilifer* Brid. and *Porella navicularis* (Lehm.&Lindenb.)Lindb. Fresh thalli were used for the whole study.

Isolation and Assay of Phenylalanine ammonia lyase (PAL) and Peroxidase (POX) - PAL was isolated from the fresh tissue following the method of Morrison *et al.*[5] and assayed by the method of Whetten and Sederoff[6]. The activity was related to the amount of cinnamic acid formed by the action of the enzyme on the substrate. One unit of PAL activity is equivalent to the µg of cinnamic acid released by the deamination of L- phenylalanine under ambient condition. Peroxidase (POX) was isolated and assayed by the method of Ingham[7] and Goliber[8] respectively. One unit of POX activity is the amount of enzyme required to oxidase 1 µmol of guaiacol by H_2O_2 at 470 nm in test condition.

Extraction and Assay of Peroxidase - Cytosolic and Cell Wall Bound - Soluble (cytostolic) and ionically bound (cell wall bound) peroxidase were isolated from fresh tissues by following the method of Alcazar *et al.*[9].

Isolation and Assay of Polyphenol Oxidase (PPO) - Polyphenol oxidase was extracted and assayed according to the method of Oktay *et al.*[10]. The activity of PPO was determined spectrophotometrically by recording the increase in absorbance at 420 nm for 10 min. One unit of the enzyme activity is defined as the amount of enzyme that caused a unit change in absorbance per min at 475 nm.

Detection of Peroxidase Under Light Microscope - Peroxidase was localized in the fresh microtome sections by using DAB following the method of Gahn[11] with appropriate controls.

Cellular Localization of Lignin - Lignin in sections of the samples were detected by Weisner and Maule's colour reaction test using 1% fluroglucinol in 70% ethanol for 3 min. The incubated sections were treated with concentrated HCl for 5 min and mounted in glycerine jelly for detecting lignin[11].

Estimation of Lignin, Total Soluble Phenol and Phenolic Acids - Lignin was extracted and quantified spectrometrically at 280 nm using dehydroconiferyl alcohol polymerizate as standard[12]. Total phenol was estimated by the method of Mayr *et al.*[13]. Phenolic constituents from the tissues were fractionated by RP-HPLC following the method of Beta *et al.*[14] and quantified by using appropriate standards. Protein content was determined by Bradford method. The data was analyzed by students 't' test followed by ANOVA and the level of significance was expressed as $P< 0.01$.

Results and Discussion

Phenolic compounds are secondary metabolites found in many plant species. They play many roles including lignin synthesis[15]. Even though, the lignin acts as structural polymer that gets synthesized enzymically, the environmental impact on its synthesis has not been revealed fully so far. Very little information is available regarding lignin or lignol like compound formation in bryophytes. The two members expressed a high profile of phenol at the range of 1.44 to 2.4 mg/g tissue (Fig. 1). Subsequent to the quantification, the total soluble phenols was further fractionated by RP-HPLC to understand the pool of phenolic acids in the thalli of *Campylopus pilifer* and *Porella navicularis*. The phenolic extracts of tissues contain the peaks of most of the standards, indicating the functional compartmentation of phenolic acids during plant growth (Fig.2a and b). The amount of major phenolic acids involved in lignin formation such as cinnamate, caffeate and ferulate were quantified effectively interpreted from the standards (Table 1). The *in vitro* phenol content and rich pool of phenolic acids in thallus was compared with lignin content. A negative correlation was observed between phenolic acids and lignin content i.e. high phenolic acids and no lignin content suggesting the non involvement of phenolic acids towards lignin formation. The analytical data of no lignin content was supported by

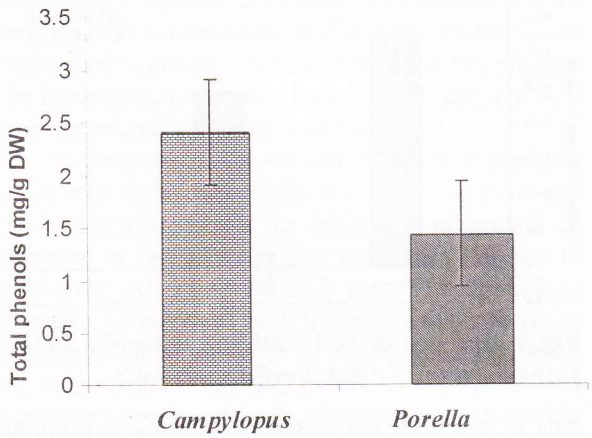

Fig.1. Total phenol content in *Campylopus pilifer* and *Porella navicularis.*

the negative response in histochemical localization of lignin. The accumulation of phenols such as cinnamate, coumerate, hydroxybenzoate, chlorogenate, caffeate, ferulate, gallic acid and paracatechol and their oxidation and condensation products including quinines, melanins and suberins may be the unknown contributors to plant resistance[16]. This accumulation of phenolics in tissue seems to give host - plant resistance towards the pathogen such as inhibiting bacterial growth by inactivating cell wall degrading enzymes and / or as precursors in the formation of physical barriers or exhibits specificities against a particular pathogen or inhibit enzyme involved in pathogenesis. Such as polygalacto uronase, glucagon synthetase, pectinase etc. or react with proteins to form tanninferous compounds[17].These suggest the possibility of synergism between structurally related phenolic compounds in imparting resistance against pathogens.

Table 1. Phenolic acids in *C.pilifer* and *P.navicularis* (μg/g tissue).

	Pcat	Cou	Caf	HBA	Chlor	Gal	Cinn	Fer
Campylopus	1.79	1969	44.7	4.37	247.9	198.3	78.7	719.5
Porella	-	19733.7	30.4	18.5	712	413.8	492.7	–

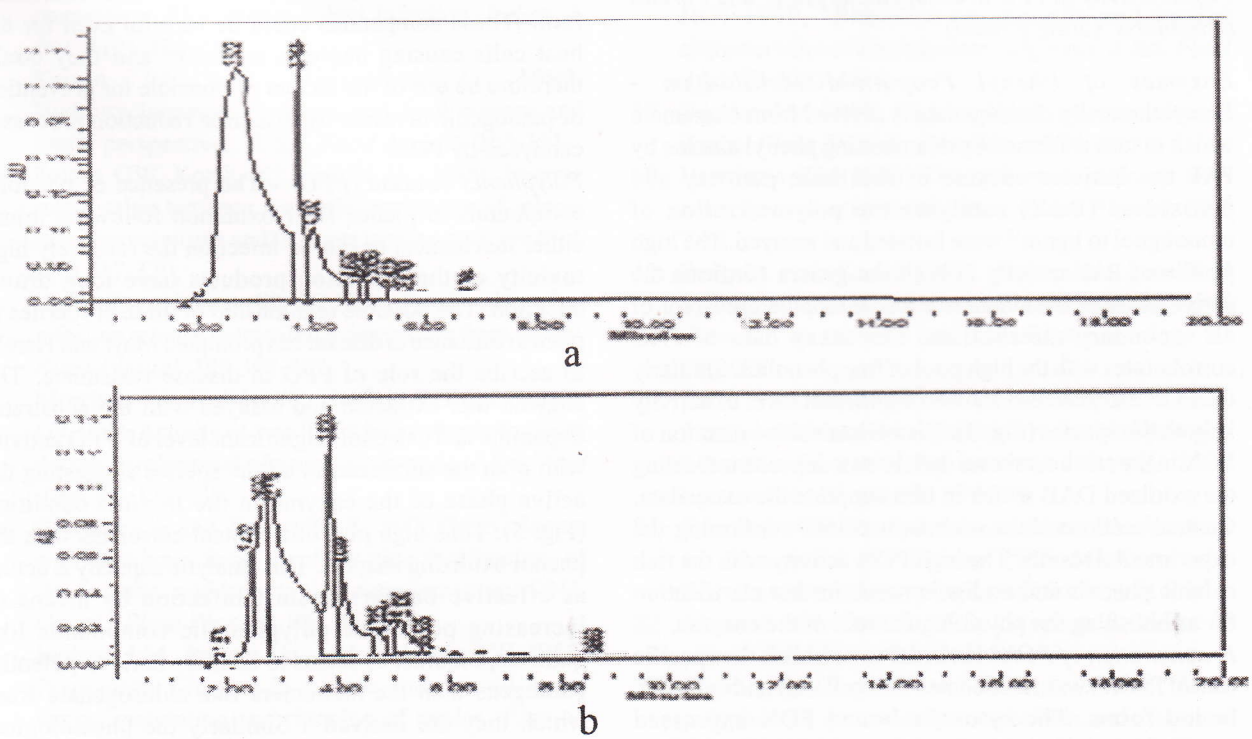

Fig.2. RP-HPLC profile of (a) *Campylopus pilifer* and (b) *Porella navicularis.*

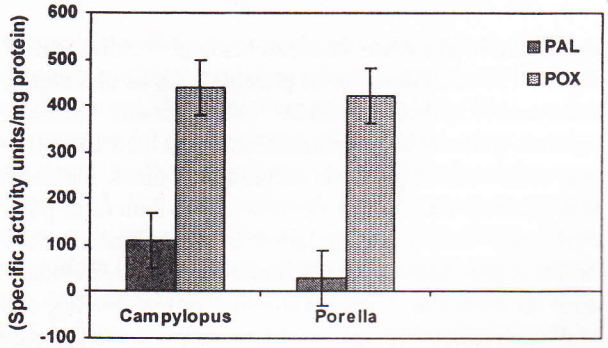

Fig.3. Activities of PAL and POX in *Campylopus pilifer* and *Porella navicularis.*

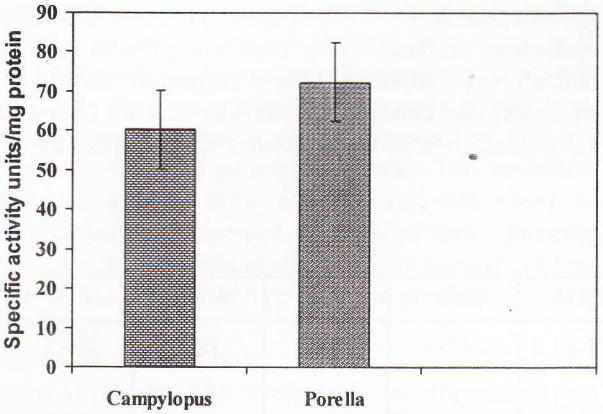

Fig.5. Activity of PPO in *Campylopus pilifer* and *Porella navicularis* (µ/mg protein)

Enzymes of Phenyl Propanoid Metabolism - Biosynthetically chlorogenate is derived from cinnamate which in turn is formed by deaminating phenyl alanine by PAL the initiator enzyme of shikimate pathway and peroxidase (POX) catalyses the polymerization of monolignol to lignin[18] were isolated and assayed. The high profile of PAL activity in both the genera confirms the active phase of the cinnamate formation, the precursor of all secondary metabolites. The assay data of PAL corroborates with the high pool of free phenolics. Similarly the POX enzyme also showed significant level of activity in both the species (Fig. 3). Histochemical localization of POX in the sections shows dark brown deposits indicating the oxidized DAB which in turn supports the assay data. Control sections show no brown colour confirming the experimental results. The high POX activity with the rich soluble phenols and no lignin needs further clarification for establishing the physiological role of the enzyme.

Fractionation of POX - In order to establish the specific role of POX it was fractionated into cell wall and cytosolic bound forms. The cytosolic bound POX expressed maximum activity than cell wall bound POX (Fig. 4). This

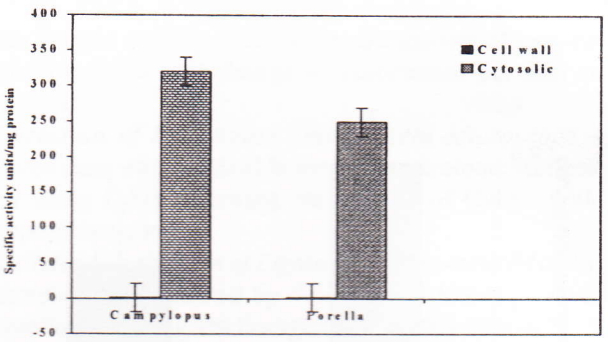

Fig.4. Activity of cell wall and cytosolic POX in *Campylopus pilifer* and *Porella navicularis.*

data makes clear the cytosolic POX has a prominent physiological role other than lignification; while the poor assay data of cell wall bound POX supports the nonlignified nature of the cells. It is possible to explain the high POX activity in bryophytes as a part of anti oxidative system against oxidative stress or as a part of general defense mechanism. The defence mechanism include the formation of oxidized state in the cell, which could cause the direct generation of ROS -H_2O_2 directly followed by the accumulation of oxidized metabolites that acts as anti microbial agents[19]. Another important function is the generation of antimicrobial reactive free radical[20] and / or the formation of other ROS that act in an immediate form .These compounds could be harmful even for the host cells causing necrosis in them[21] and they could therefore be one of the factors responsible for prevention of pathogenic invasion by oxidation reduction processes catalysed by POX.

Polyphenol oxidase (PPO) - The presence of phenolic compounds in plants, their oxidation following injury either mechanical or due to infection the relatively high toxicity of the oxidation products have long drawn attention. The possible relationship of these properties to plant's resistance to disease has prompted Mayr and Harel[22] to ascribe the role of PPO in disease resistance. The enzyme was extracted and assayed with the substrates dopamine and catechol. Significant level of PPO activity with both the substrates in all the species suggesting the active phase of the enzyme in the *in vitro* condition (Fig. 5). Thus high phenolic content correlates with the phenol oxidizing enzyme. This catalytic capacity is acting as effective barriers against infection by means of increasing polymeric polyphenolic compounds like quinines, diquinones seems to be more toxic to potential pathogens than the monomers like chlorogenate from which they are derived[16]. Similarly the physiological effects of polyphenols are considered to be that of

astringency based on their ability to complex with proteinaceous compounds. These may render tissue unpalatable to a predator by precipitating salivary proteins or by immobilizing enzymes, impede the invasion of host tissues by microbial predators or parasites[23].

In conclusion phenolic compounds in bryophytes may be regarded as potentially toxic compounds, which may be associated with the inhibition of growth of pathogens or reduction in ther multiplication rate in bryophytes. Alternatively they may be regarded as substrates of enzymes which convert them to other compounds which are more directly related to disease resistance. A preliminary attempt was made in the selected bryophytes regarding the role of phenolics and their role as defense. The active phase of PAL, rich cinnamate supports the overview. The high profile of cytosolic POX probably plays the scavenging role against the H_2O_2 or creating an oxidized state in the cell that act as antipathogenic. Even though the whole study reveals a basic concept on the biochemical defense mechanism in bryophytes, ultrastructural studies are warranted to establish polyphenols in bryophytes and their role in defense.

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