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Phenolic Acid Changes in Mycelia of *Sclerotium rolfsii* After Garlic and Onion Supplementation in a Broth Medium

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High performance liquid chromatographic (HPLC) analysis of mycelia of *Sclerotium rolfsii* grown in broth medium supplemented with garlic (*Allium sativum*) and onion (*Allium cepa*) was carried out to estimate qualitative and quantitative changes in phenolic acids. Several phenolic acids, such as gallic, chlorogenic, ferulic, *o*-coumaric and cinnamic acids were detected in varied amounts in mycelia grown on such media as compared to control. Phenolic acids represents a wide range of secondary metabolite found in the cells of plants and microbes including fungi. The growth characters of *S. rolfsii* in various supplements also varied from thin and transparent to thick and opaque.

KEYWORDS: ***Allium cepa*, *Allium sativum*, Phenolic acids, *Sclerotium rolfsii***

The genus *Sclerotium* as described by Saccardo (Saccardo, 1913) includes fungi that form sclerotia and sterile mycelia. Several fungi from different groups are included in the genus, which are characterized by the production of small, tan to dark brown or black spherical sclerotia internally differentiated into rind, cortex and medulla (Punja and Rahe, 1992). *Sclerotium rolfsii* Sacc. is the best known member of the genus found ubiquitously as a destructive plant pathogen (Aycock, 1966; Punja, 1985; Sarma *et al.*, 2003). The pathogen has extensive host range because of its prolific growth rate and production of cell degrading enzymes.

Today, biocontrol agents are gaining much popularity for the control of plant diseases. However, their effects on the physiology of fungal pathogens are not known. The purpose of this study was to analyze the qualitative and quantitative changes in phenolic acids in the mycelia of *S. rolfsii* as influenced by leaves, immature and mature bulbs of garlic (*Allium sativum*) and onion (*Allium cepa*). Phenolic acids represent a wide range of secondary metabolic compounds found in the cells of plants and microbes including fungi. They are regarded to impart active defense responses. In *S. rolfsii*, it is assumed that phenolic acid protects this fungus from the attack of other soil inhabiting microbes (Sarma *et al.*, 2002). Since garlic and onion are well known plants that have antimicrobial property, in this study it is used as low nutrition medium to see phenolic acid changes of fungal mycelia grown in broth medium.

Materials and Methods

Isolation and maintenance of the fungal culture. The isolate of *S. rolfsii* causing collar rot in chickpea (*Cicer arietinum*) used in the study was collected from the agricultural farm of Banaras Hindu University, Varanasi, India. The fungus was isolated by inoculating sclerotia from the infected parts of the plant in Petri dishes containing potato dextrose agar (PDA) (peeled potato 200 g, dextrose 20 g, agar 15 g, distilled water 1 *l*) medium and incubating the plates at 25±2°C for 7~10 days till sclerotium formation. From each plate single sclerotium was taken out and inoculated onto PDA slants. In this way the isolates were purified and such slants were kept at 4°C for further use.

Growth characters of mycelial mat in broth media. The present experiments were conducted with fresh leaves and bulbs of two-month-old garlic (*A. sativum*) and onion (*A. cepa*), their roots, mature garlic clove and mature red and white onion bulbs. All the plant materials were cleaned by washing in distilled water and air-dried to remove trace water from the surface. They were crushed in a pestle and mortar to make a paste. Such pastes were used at different concentrations (1, 3, 5% w/v) prepared in distilled water in conical flasks (100 *ml*) without agar in the medium (30 *ml* in each conical flask). The medium was sterilized in autoclave at 121°C for 10 minutes. A five millimeter (dia) mycelial disc from 4-day-old cultures of *S. rolfsii* grown on PDA medium was inoculated into the liquid medium in flasks with the help of a sterilized inoculation needle. The flasks were incubated at 25±2°C and observed for growth characters of mycelial mat after 10 and 20 days. The potato dextrose broth (PDB; peeled potato 200 g, dextrose 20 g, distilled water 1 *l*) which was similarly inoculated and incubated served as control. The growth characters of mycelial mat were usually recorded in all the treatments. The experiment was conducted in triplicate and repeated twice.

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Extraction of mycelia for phenolic acids. Thirty milliliter of broth medium supplemented with different concentrations (1, 3, 5% w/v) of the paste prepared in distilled water was poured into conical flasks (100 *ml*) and mycelial bits from exponentially growing culture of *S. rolfsii* were inoculated separately. Cultures were allowed to grow for 10 and 20 days at 25±2°C as described earlier. The mycelial mat developed in broth media was harvested on sterilized Whatman No. 1 filter papers after 10 and 20 days and washed three times in distilled water and then placed on a pad of sterile filter paper to remove excess water. Two gram of fresh mycelial mat from each treatment and each concentration of the paste was thoroughly macerated separately in the presence of ethanol : water (80 : 20, v/v) in a pestle-mortar and collected in screw-capped bottles and kept overnight. The extract was fractionated with equal volume of ethyl acetate. The ethyl acetate fraction was evaporated under vacuum (Buchi Rotavapor Re Type) to dryness and the material was dissolved in 1.0 *ml* methanol (HPLC grade) for further analysis.

HPLC analysis. High performance liquid chromatography (HPLC) of the samples was performed with an HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with two Shimadzu LC-10 AT*VP* reciprocating pumps, a variable UV-VIS detector (Shimadzu SPD-10 A*VP*) and a Winchrom integrator (Winchrom). Reverse phase chromatographic analysis was carried out in isocratic conditions using RP C-18 HPLC column (250 × 4.6 mm id, particle size 5 *μ*m, Luna 5 *μ* C-18 (2), Phenomenex, USA) at room temperature. Running conditions included injection volume: 5 *μl*, mobile phase: methanol-0.4% acetic acid (80 : 20, v/v), flow rate: 1 *ml*/min, detection at 290 nm and attenuation response 0.03 AUFS. Samples were filtered through membrane filters (Pore size 0.20 *μ*m, Millipore) prior to injection in a sample loop. Gallic, ferulic, caffeic, tannic, chlorogenic, *o*-coumaric and cinnamic acids were used as internal and external standards. Phenolic compounds present in the samples were identified by comparing retention time (Rt) of the standards and by the co-chromatography. Contents of phenolic acids were calculated by comparing peak areas of reference compounds with those in the samples run under similar elution conditions. Analyses were made three times from each sample and mean value was calculated for each compound. Mean relative concentrations of phenolic compounds were statistically analyzed by ANOVA and the normality of the results and homogeneity of the variances were tested.

Results and Discussion

The best mycelial growth was obtained in potato dextrose broth and in higher concentration (i.e., 5%) of paste-added broth medium of all the treatments except mature garlic clove. Mostly mycelial mat of *S. rolfsii* was thicker and opaque in 5% in comparison to 1 and 3%, where it was thin and transparent/translucent after 10 and 20 days of inoculation. The fungus grew in only 1% concentration of paste of mature garlic clove as thin and translucent after 10 days which became thick and opaque after 20 days of inoculation. There was no growth up to 20 days of inoculation in 3 and 5% mature garlic clove paste. Similarly, there was no growth in two-month-old garlic root even at 1% concentration. Formation and/or maturation of sclerotia differed in each concentration after 10 and 20 days of inoculation. However, there was no definite pattern of growth characters and sclerotium formation/maturation (Table 1). Basidial stage induction was observed in 3% garlic leaves after 15 days of inoculation in the center of the medium as compact, whitish, thick growth, which revealed the presence of basidial hymenia on microscopic observation and basidiospores formed singly on each of the four sterigmataof a basidium. The basidia measured was 10~12 × 4~5 *μ*m and basidiospores 3~5 × 2~4 *μ*m (Fig. 1a-b).

HPLC analysis of mycelial mat of *S. rolfsii* obtained from different concentrations of different treatments revealed consistently presence of 5 phenolic acids which were identified on the basis of their retention time (Rt.) as well as by co-injection with the standard compounds. The compounds identified were as gallic (Rt.2. 86 min), ferulic (Rt. 3.42 min), *o*-coumaric (Rt. 3.70 min), chlorogenic (Rt. 4.16 min) and cinnamic acids (Rt. 4.46 min) (Fig. 2). Mycelial mat obtained from potato dextrose broth revealed the presence of 0.14 *μ*g/g chlorogenic (Chl.A), 1.01 *μ*g/g ferulic (FA) and 0.05 *μ*g/g *o*-coumaric acids (Oc.A) after 10 days of inoculation while 2.23 *μ*g/g gallic (GA), 3.20 *μ*g/g ferulic, 1.24 *μ*g/g *o*-coumaric and 0.13 *μ*g/g cinnamic acids (Cin.A) after 20 days of inoculation. After treatment with 1% paste of two-month-old garlic leaves, 13.22 *μ*g/g FA was detected in 10-day-old mycelia whereas 2.38, 0.60, 6.27 and 0.02 *μ*g/g GA, Chl.A, Oc.A and Cin.A after 20 days, respectively. Ferulic acid increased with increasing concentrations of leaf paste in mycelial mat harvested after 10 days of inoculation. In two-month-old garlic bulbs, 21.35 *μ*g/g FA was detected in 10-day-old mycelia treated with 1% bulb paste, 2.03 *μ*g/g GA, 1.00 *μ*g/g FA and 0.58 *μ*g/g Oc.A was detected after 20 days. 1.61 *μ*g/g Chl.A and 3.38 *μ*g/g FA were present in 10-day-old mycelial mat treated with 3% bulb paste while 1.01 *μ*g/g GA, 0.67 *μ*g/g Chl.A, 0.75 *μ*g/g Oc.A and 0.01 *μ*g/g Cin.A were present in 20-day-old mycelia. Mycelial mat obtained from 5% bulb paste revealed 1.03 *μ*g/g FA and 0.02 *μ*g/g Oc.A after 10 days. In 1% mature garlic clove treatment 1.41 *μ*g/g Chl.A, 321.21 *μ*g/g FA and 19.95 *μ*g/g Oc.A were detected in 10-day-old myce-

Table 1. Growth characters of mycelial mat of *Sclerotium rolfsii* after garlic and onion application in broth medium

Treatment with	After 10 days	After 20 days
PDB	Very thick, white mycelial mat, no sclerotia	Very thick, compact & white mycelial mat, no sclerotia
2MOGL		
1%	Very thin, transparent mycelial mat, 5~7 white immature sclerotia/flask	Very thin, translucent mycelial mat, 8~9 dark brown mature sclerotia/flask
3%	Slightly thick, white, translucent mycelial mat, no sclerotia	Thick, white, opaque mycelial mat, 10~15 mature dark brown sclerotia/flask
5%	Slightly thick, white, translucent mycelial mat, no sclerotia	Very thick, white mycelial mat, 15~17 mature dark brown sclerotia/flask
2MOGB		
1%	Very thin, transparent mycelial mat, no sclerotia	Very thin, transparent mycelial mat, 10~12 mature dark brown sclerotia/flask
3%	Thin, translucent mycelial mat, no sclerotia	Thin, translucent mycelial mat, 40~50 mature dark brown sclerotia/flask
5%	Thick, white mycelial mat, 85~95 mature dark brown sclerotia/flask	Thick, white mycelial mat, 95~100 mature dark brown sclerotia/flask
MGC		
1%	Thin, translucent mycelial mat, no sclerotia	Thick, white, opaque mycelial mat, 50~60 mature dark brown sclerotia/flask
2MOROL		
1%	Very thin, transparent mycelial mat, no sclerotia	Very thin, transparent mycelial mat, 20~25 dark brown sclerotia/flask
3%	Thin, translucent mycelial mat, 10~12 light brown sclerotia/flask	Thin, transparent mycelial mat, 20~30 dark brown sclerotia/flask
5%	Thick, white, opaque mycelial mat, 20~25 light brown sclerotia/flask	Thick, white, opaque mycelial mat, 40~50 mature dark brown sclerotia/flask
2MOROB		
1%	Very thin, transparent mycelial mat, 10~12 light brown sclerotia/flask	Very thin, transparent mycelial mat, 20~25 dark brown sclerotia/flask
3%	Thin, translucent mycelial mat, 10~12 light brown sclerotia/flask	Thin, translucent mycelial mat, 30~35 dark brown sclerotia/flask
5%	Thick, white, opaque mycelial mat, 20~25 light brown sclerotia/flask	Thick, white, opaque mycelial mat, 40~50 mature dark brown sclerotia/flask
MROB		
1%	Thin, transparent mycelial mat, no sclerotia	Thin, transparent mycelial mat, no sclerotia
3%	Thin, transparent mycelial mat, no sclerotia	Thick, opaque mycelial mat, no sclerotia
5%	Thick, opaque mycelial mat, no sclerotia	Thick, opaque mycelial mat, no sclerotia
MWOB		
1%	Thin, transparent mycelial mat, no sclerotia	Thin, transparent mycelial mat, no sclerotia
3%	Thick, opaque mycelial mat, no sclerotia	Thick, opaque mycelial mat, no sclerotia
5%	Thick, opaque mycelial mat, no sclerotia	Very thick, opaque mycelial mat, no sclerotia

PDB= Potato dextrose broth, 2MOGL = 2 Month old garlic leaves, 2MOGB = 2 Month old garlic bulbs, 2MOROL = 2 Month old red onion leaves, 2MOROB = 2 Month old red onion bulbs, MROB = Mature red onion bulbs, MWOB = Mature white onion bulbs.

lial mat while 0.17 μg/g GA, 0.06 μg/g Chl.A, 1.20 μg/g Oc.A and 0.07 μg/g Cin.A in 20-day-old mycelia. In two-month-old red onion leaves, 1.79 μg/g GA, 0.25 μg/g FA and 0.13 μg/g Oc.A in 1% concentration; 0.23 μg/g GA, 0.09 μg/g Chl.A, 0.06 μg/g FA and 0.02 μg/g Oc.A in 3%; 0.26 μg/g GA, and 0.07 μg/g Oc.A in 5% and 0.01 μg/g Cin.A were detected in all the three concentrations (1, 3 and 5%) in 10-day-old mycelia whereas 4.95 μg/g GA, 0.48 μg/g FA, 0.96 μg/g Oc.A in 1%; 0.89 μg/g GA, 0.37 μg/g ChlA, 0.18 μg/g FA, 0.30 μg/g Oc.A in 3%; 2.22 μg/g GA, 0.62 μg/g Chl.A, 0.34 μg/g FA, 0.15 μg/g Oc.A in 5% in 20-day-old mycelial mat. Different amounts of phenolic acids were detected in mycelia grown in different concentrations of two-month-old red onion bulbs, mature red and white onion bulb paste (Table 2).

The mycelia of *S. rolfsii* contain some phenolic acids which seem to be needed for their growth and resistance against adverse conditions of the environment. Following treatment with garlic and onion, the amount of these phenolic acids increased in comparison to control (potato dextrose broth only) when observed after 10 days of inoculation. In some cases they were found less than control. Both the plants (garlic and onion) were able to increase the synthesis of phenolic acids in *S. rolfsii* as compared to

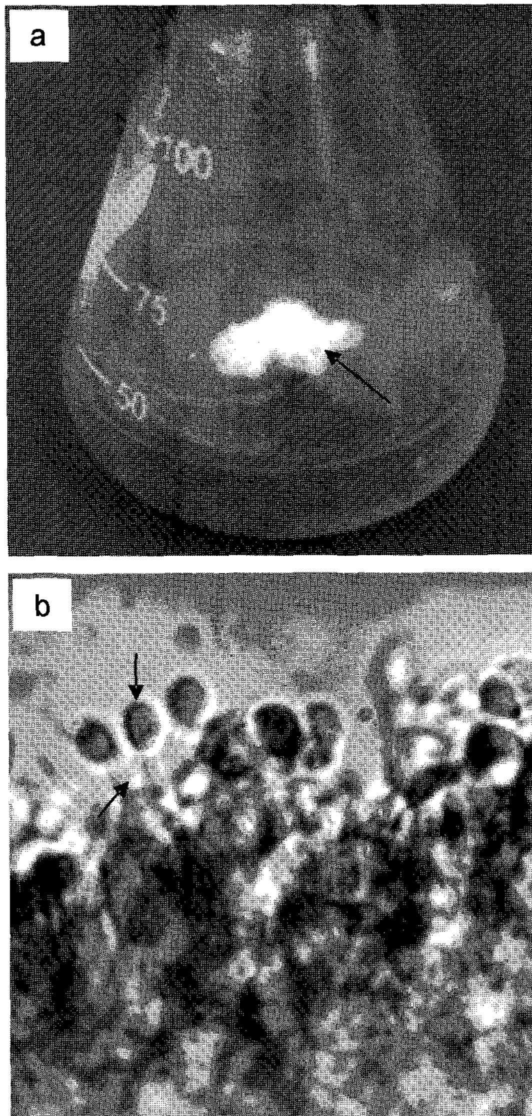

Fig. 1. Basidial stage induction in chickpea (*Cicer arietinum*) isolate of *Sclerotium rolfsii* in broth medium of garlic leaves. (a) Basidial stage induction in broth medium (arrow), original size, (b) Basidia and basidiospores (arrow), ×1000.

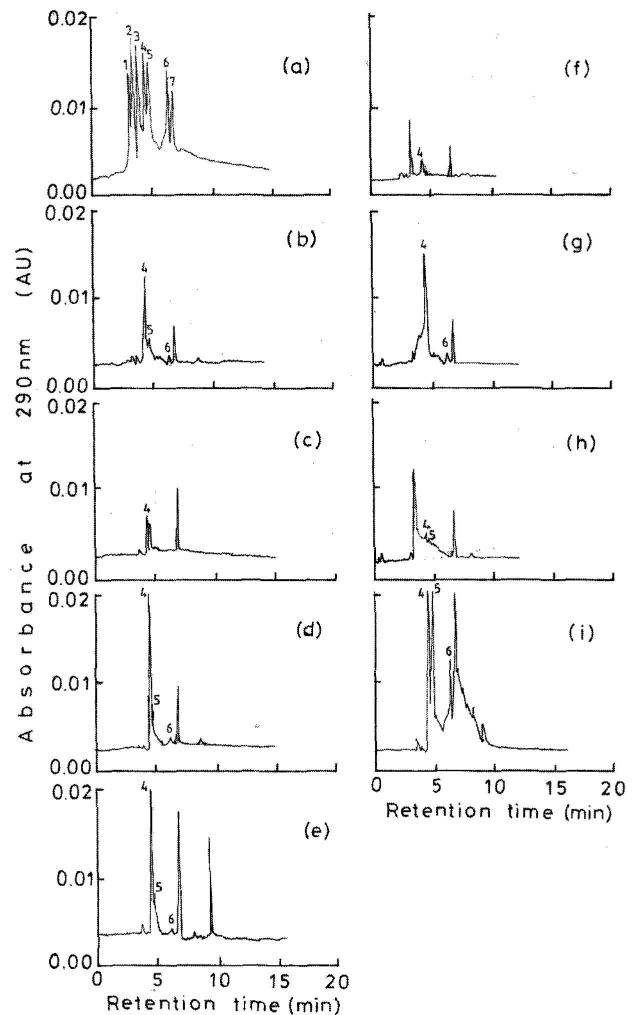

Fig. 2. Phenolic profile of 10 days old mycelia of *Sclerotium rolfsii* obtain from different treatments of garlic, (a) Peaks of reference phenolic compounds, Mycelia from (b) potato dextrose broth, (c) 1% (d) 3% (e) 5% treatment of two month old garlic leaves, (f) 1% (g) 3% (h) 5% treatment of two month old garlic bulb (i) 1% mature garlic clove treatment (peak nos. 1 = tannic, 2 = gallic, 3 = vanillic, 4 = ferulic, 5 = *o*-coumaric, 6-chlorogenic, 7 = cinnamic acid).

control. The phenolic acids were found to be decreased in 20-day-old mycelial mat in some cases. This may be due to conversion of one phenolic acid into another. Increase of phenolic acid was also observed in different concentrations after 20 days. Interestingly, the growth of mycelial mat was thin in lower than higher concentration and control. This may be due to less amount of nutrients present in lower concentration which affect the growth. Inhibition of growth of *S. rolfsii* in 3% and 5% of mature garlic clove paste indicates its antifungal nature at these concentrations. Inhibition of growth in all concentrations of two-month-old fresh garlic root may be due to antifungal nature or unavailability of nutrients in proper amounts.

Results of the present investigation reveal interesting observations in relation to mycelial growth and presence of different phenolic acids in mycelia of *S. rolfsii* under the influence of different parts of garlic and onion. The presence of high amount of ferulic acid in mycelia does not appear to affect growth and development of *S. rolfsii* itself. Sarma and Singh (2003) reported that ferulic acid could inhibit complete mycelial growth of *S. rolfsii* at a concentration of 1000 μg/g. Since the maximum concentration of ferulic acid in mycelia obtained from 1% mature garlic clove treatment was 321.21 μg/g, it becomes very difficult to interpret as to whether only ferulic acid is acting as inhibitory agent or combination of several other compounds including ferulic acid for the said purpose.

Table 2. Phenolic acids (μg/g mycelial fresh weight) profile in mycelia of *Sclerotium rolfsii* after garlic and onion application in a broth medium

Treatment with	Phenolic acid (μg/g mycelial fresh weight)									
	After 10 days					After 20 days				
	GA	Chl. A	FA	Oc.A	Cin.A	GA	Chl. A	FA	Oc.A	Cin.A
PDB	ND	0.14	1.01	0.05	ND	2.23	ND	3.20	1.24	0.13
2MOGL										
1%	ND	ND	13.22	ND	ND	2.38	0.60	ND	6.27	0.02
3%	ND	2.01	114.21	0.58	ND	1.49	ND	0.31	1.91	0.07
5%	ND	0.53	124.64	0.20	ND	2.03	1.46	0.05	3.77	0.01
2MOGB										
1%	ND	ND	21.35	ND	ND	2.03	ND	1.00	0.58	ND
3%	ND	1.61	3.38	ND	ND	1.01	0.67	ND	0.75	0.01
5%	ND	ND	1.03	0.02	ND	1.43	1.34	ND	0.35	0.02
MGC										
1%	ND	1.41	321.21	19.95	ND	0.17	0.06	ND	1.20	0.07
2MOROL										
1%	1.79	ND	0.25	0.13	0.01	4.95	ND	0.48	0.96	ND
3%	0.23	0.09	0.06	0.02	0.01	0.89	0.37	0.18	0.30	ND
5%	0.26	ND	ND	0.07	0.01	2.22	0.62	0.34	0.15	ND
2MOROB										
1%	0.62	ND	0.05	ND	0.01	2.99	ND	0.34	0.10	ND
3%	0.97	ND	0.06	0.01	0.01	3.02	ND	0.08	0.09	ND
5%	0.88	0.12	0.13	0.06	ND	1.04	ND	0.20	0.09	ND
MROB										
1%	1.76	0.80	0.25	0.09	0.01	0.51	0.92	ND	0.14	0.01
3%	0.46	0.30	ND	ND	0.02	0.93	0.65	ND	0.09	ND
5%	0.31	0.45	0.12	0.07	0.01	0.28	0.49	ND	0.09	0.03
MWOB										
1%	1.02	0.52	ND	0.10	0.01	1.15	0.68	ND	0.12	0.01
3%	0.14	0.23	0.05	0.03	0.01	0.21	0.31	0.02	0.02	0.07
5%	1.45	1.00	ND	0.13	0.01	1.41	0.92	ND	0.18	0.01

PDB = Potato dextrose broth, 2MOGL = 2 Month old garlic leaves, 2MOGB = 2 Month old garlic bulbs, 2MOROL = 2 Month old red onion leaves, 2MOROB = 2 Month old red onion bulbs, MROB = Mature red onion bulbs, MWOB = Mature white onion bulbs, ND = Not detected, GA = Gallic acid, Chl.A = Chlorogenic acid, FA = Ferulic acid, Oc.A = *o*-Coumaric acid, Cin.A = Cinnamic acid.

Considering the effects of the two herbals on mycelial growth, leaves supported better growth as compared to bulbs. The role of phenolics as secondary metabolites in living organism is to induce resistance for protection of life besides several other activities (Harborne, 1988). As some of them are also functionally anti-oxidants, the senescence of the living being is expected to be prolonged. It has also been generally observed that during non-balanced growth of microbial cell due to high accumulation of precursors like acetate, malonate, pyruvate etc. cells die but the toxicity of these precursors is said to be detoxified by the secondary metabolites (Weinberg, 1970; Woodruff, 1966). The better growth of *S. rolfsii* on leaf paste medium may be due to the presence of high amount of simple sugars in leaves than bulbs. A detailed study is needed to confirm these observations.

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