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Atlantic Research Corporation
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Subject: Synthesis of Rubber by Microorganisms
Status Report - 1 January to 31 March, 1956
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Summary

Two of the mutants derived from *Lactaria subdulcis* (P) by irradiation with electrons were grown in submerged culture, using a two-week culture period, until sufficient mycelia were harvested for extraction and identification of rubber. The extractions are underway.

The clear, aqueous filtrates from these cultures, after separation of mycelia, were shaken with benzene, and the benzene extracts reduced under nitrogen to small volume. The aqueous phase yielded 0.67% (based on dry weight of mycelia) of a "sticky", "leggy", rubbery polymer. The polymer was insoluble in water, alcohol, and acetone, and was not hydrolyzed by hot alkali. The material was submitted to Dr. McLaughlin of the Walter Reed Hospital for infra-red analysis. This is the first evidence of extra-cellular production of rubber by species of *Lactaria*. Lysis of cells as the source of polymer is considered improbable since the culture period was short and the quantity of elastomer relative to the total expected from the mycelia was large. Filtrates from the culture of other species of *Lactaria* when extracted with benzene have yielded no "rubbery" polymer.

Lactaria mucida, which grows slowly at temperatures above 23°C, is being cultivated on the shaker. Production of sufficient mycelia for extraction and subsequent examination for the presence or absence of rubber is expected by the middle of April.

Experiments designed to determine the feasibility of direct extraction of mycelia with benzene are in progress. Mycelia from still cultures of *L. subdulcis* (P) were used for this study.

Pure cultures of the following species of *Lactaria* were sent to Dr. Leonard for the type culture collection and to Dr. Tsuchiya of the Northern Regional Laboratories (USDA): *L. paludinella*, *L. piperata*, *L. subdulcis*, *L. subdulcis* (P), *L. torminosa*. Pure cultures of other species of interest, including the irradiated mutants, will be mailed in April.

Since cyclitols are the predominant carbohydrates of *Hevea* and *Lactaria* the species of *Lactaria* of our collection were characterized on media based on mannitol. Mannitol, which occurs in the sporophores of *Lactaria*, was substituted for dextrose in the Sabouraud medium.

Experimental

Cultivation, Separation, and Extraction of Mycelia of the Mutants Obtained from L. Subdulcis (P) by Irradiation with Electrons

The mutants obtained from *L. subdulcis* (P) by irradiation with electrons, i.e., *L. subdulcis* (P- $\gamma\beta$) induced by direct irradiation under the 2,000,000 volt beam from a Van de Graaff Electrostatic generator at a dosage of 1×10^6 rep, and *L. subdulcis* (P- γ) induced by indirect irradiation at the same dosage, were cultivated on the shaker to get material for extraction and the determination of their ability to produce rubber on liquid, synthetic media. Resolution of the feasibility of this approach for the production of high-yielding, rubber-producing strains of fungi before terminating the research program was deemed important.

Erlenmeyer flasks containing 100 ml. of an aqueous medium consisting of 2.5% Fleischmann's Diamalt syrup, 0.5% malt extract and 0.5% neopeptone were inoculated with the mutants, using 5% inoculum. The pH of the medium after autoclaving was 5.0. Twenty flasks of each mutant were placed on the shaker and cultivated for a period of two weeks at temperatures ranging from 22 - 25°C. The cultures were then removed, the mycelia separated from the media by filtration, quick-frozen at -18°C, and preserved in three volumes of hot ethanol containing hydroquinone (.040 gms/liter) as antioxidant. This process was repeated until approximately 80 grams (dry wt. basis-calc.) of mycelia of each mutant were harvested. Sixteen liters of culture medium, for each mutant, were required.

The clear filtrates from the cultures of the mutants, after separation of mycelia, were combined and extracted with benzene. Five hundred milliliter aliquots of the filtrates were shaken first with 100 milliliters of benzene in a separatory funnel, and then with 75 milliliters of solvent. The benzene extracts from 28 liters of medium were combined and reduced to a volume of 100 milliliters. Solvent was removed with a stream of oxygen-free nitrogen. Phenyl- β -Naphthylamine, 0.1% was added as antioxidant. An aliquot showed the benzene solubles to be 0.947 gms or 0.68% on the mycelia (138 grams-dry wt.) produced by the media. The residue from the aliquot was a sticky, "leggy", rubbery polymer. It was not soluble in water, alcohol, or acetone. The remaining benzene solution blanketed with nitrogen was submitted to Dr. McLaughlin of Walter Reed Hospital for infra-red analysis.

Response Variation of Species of Lactaria
on Media with Mannitol as the Carbohydrate

Cyclitols, quebrachitol (or inositol) in Hevea and mannitol in Lactaria, are the predominant carbohydrates of tissues of these rubber-bearing plants. These carbohydrates may have a role in rubber synthesis. The species of Lactaria, including the mutants, were characterized as to response on the following media:

(a) Mannitol agar

Neopeptone	5 gm
d-Mannitol	20 gm
Bacto agar	7.5 gm
Deionized water	500 ml
pH after sterilization	6+

Note: This is the Sabouraud medium with mannitol substituted for dextrose.

(b) Mannitol-Diamalt Agar

Neopeptone	2.5 gm
Fleischmann's Diamalt	7.5 gm
d-Mannitol	7.5 gm
Bacto-agar	7.5 gm
Deionized water	500 ml
pH after sterilization	5+

With Fleischmann's Diamalt syrup as a source of accessory food factors and minerals the Lactaria are readily adapted for growth on media containing mannitol. Growth on the mannitol agar did not differ significantly from that on Sabouraud-dextrose. The response of the various species is shown in Tables VIIIB, VIIIB, XVIII, XVIIIIB, XVIIIIC, XIX, XIXB.

Cultivation of Lactaria Mucida

Colonies of *L. mucida* grown on media containing mannitol are very rubbery in texture. Exudates from the colonies when rubbed to dryness between the fingers are "sticky and leggy". This fungus was grown in shaker culture in the following aqueous mediums:

Fleischmann's Diamalt syrup	1.0%
Mannitol	1.5%
Neopeptone	0.5%

Inoculum was 5% and period of growth at 22 - 25°C was one month. Some difficulty has been experienced with the cultivation of this species,--the cultures undergo lysis within a period of forty-eight hours when the temperature exceeds 70°F. Forty grams (dry weight) have been harvested. Sufficient material for extraction and characterization of the rubber is expected by the middle of April.

"Vistex" Extractions

Mutants (irradiated) from *L. subdulcis* (P) were grown in still culture in Fernbach flasks, using 200 mls per flask of medium consisting of 2.5% Fleischmann's Diamalt, 1.5% mannitol and 0.5% neopeptone. The mycelia were harvested, quick-frozen at -18°C , and thawed in hot ethanol. The mycelia were separated from the ethanol and extracted with benzene. Results will be presented in the final report.

Perpetuation of Cultures

Pure cultures of the following species of *Lactaria*, with instructions for culture were prepared and mailed to Dr. Leonard of NRL and to Dr. Tsuchiya of the Peoria Regional Laboratory (USDA):

- L. paludinella*
- L. piperata*
- L. subdulcis* (Black parent strain)
- L. subdulcis* (Spontaneous pink mutant)
- L. torminosa*

Pure cultures of other species will be mailed upon termination of the project in April.

Trace Element Diamalt

TABLE VII B

Response/Variation of Irradiation-Induced Mutants

Species	Diameter	Surface	Margin	Pigmentation		Reverse	Texture-Remarks	Compared to Norm
				Top	30 days			
Giant Colonies								
	9 days							
Gantiera γ	85 mm	Patches, spiky submerged	Fringed	Whitish patches	None	None	Coarse stretchy membrane, many clumps of crystals, vinegar odor	More crystals
groveicens $\gamma\beta$	85 mm	Thread like patches, wrinkled	Fringed	Whitish patches	None	None	Coarse, tacky membrane, more crystals, vinegar odor	Many more crystals more tacky
Lactaria murida γ	17 mm	Highly raised woolly	Undulate	Whitish	Brown	Brown	Chitinous, good growth	More pigment
Lactaria γ	20 mm	Spiky center glabrous	Channeled	Light pink	Light red	Light red	Thick skin like, pigment faded	Similar
subdulcis P $\gamma\beta$	20 mm	Tufted center channelled	Undulate	Pink	Orange	Orange	Thick skin like, pigment faded	Similar
Lactaria γ	50 mm	Highly raised silky	Undulate	White, watery exudate	Ochre	Ochre	Stretchy, makes legs, good growth	More rubbery, some crystals
torminosa $\gamma\beta$	45 mm	Raised, silky	Undulate halo	White, watery exudate	Pale ochre	Pale ochre	Skin like, scapy, growth submerged, pigment faded	Less rubbery, many large crystals
Lactaria γ	50 mm	Velvety channelled	Hyaline halo	Rose beige White rays, border	Pale ochre	Pale ochre	Skin like, not tacky	Less pigment
vellerea $\gamma\beta$	52 mm	Velvety	Halo	Whitish, pink ring	Pale ochre	Pale ochre	Skin like, not tacky	Very little pigment

TABLE XVIII

Response-Variation.

Species	Diameter	9 days	Giant Colonias			30 days	
			Surface	Margia	Top		Pigmentation
<i>Gautiera groveolens</i>	85 mm	Raised, hairy	Dendritic	White	White	Yellowish	Very stretchy membrane, submerged, odor of vinegar
<i>Bulgaria rufa</i>	50 mm	Raised, coarse hairy	Fringed	White	Light tan		Heavy abundant growth, spots of brown pigment, no odor
<i>Lactaria chrysotheca</i>	25 mm	Coarse spiky center	Undulate	Grey brown center, white			One plate shows possible coral pink mutant, good growth, chitinous, musty odor
<i>Lactaria paludinella</i>	53 mm	Very highly raised, cottony	Slightly irregular	White	Light ochre		Good growth, reverse ochre leathery
<i>Lactaria piperata</i>	30 mm	Highly raised silky	Wide hyaline border	White	Orange		Good growth, wide hyaline halo stretchy, skin-like mat
<i>Lactaria mucoida</i>	12 mm	Very highly raised, cottony	Channeled	Off white	Brown		Very good growth, larger mat on this medium, makes legs, mushroom odor
<i>Lactaria subdulcis</i> (B)	20 mm	Flat, smooth	Undulate	Chocolate, white border	Brown		Whitish pruinose off, wide halo tacky
<i>Lactaria subdulcis</i> (P)	22 mm	Spiky center glabrous	Channeled	Light pink, dark pink ring	Rose red pink diffuses		Good growth, pigment fades tacky
<i>Lactaria forminosa</i>	55 mm	Highly raised cottony	Entire	White	None		Good growth, reverse ochre, cottony, leathery
<i>Lactaria vellerea</i>	67 mm	Flat velvety	Undulate	Pink, darker rings	White		Good growth, stretchy mat, tacky

Mannitol-Diamant

TABLE XVIII B

Response Variation of Irradiation-Induced Mutants

Species	Diameter	Surface	Margin	Pigmentation		Texture-Remarks	Compared to Norm
				Top	Reverse		
	9 days					30 days	
Gautiera γ	85 mm	Raised, silky	Somewhat dendritic	White	Yellowish	Stretchy membrane, tacky, submerged, vinegary	Less tough membrane
groenclaus $\gamma\beta$	85 mm	Raised, fine silky	Less dendritic	White	Yellowish	Membrane tears rather than stretches	Very delicate membrane
Lactaria mucida γ	11 x 20	Raised, spiky	Crenulate	Whitish and amber	Brown	Good growth, makes legs mushroom odor	Better growth, less exudate
Lactaria γ	19 mm	Glabrous slightly channelled	Undulate	Lavender	Pink, some diffuse pink	Good growth, red reverse tacky	Better growth, more pigment, pruinose
subdulcis P $\gamma\beta$	25 mm	Glabrous channelled	Undulate	Rose	Red-diffuse light red	Very good growth, very tacky	Better growth, much more pigment
Lactaria γ	60 mm	Highly raised cottony	Entire	White	None	Good growth, reverse ochre, leathery, tacky	More pigment, more tacky
terminosa $\gamma\beta$	60 mm	Raised cottony	Entire, hyaline border	White	None	Good growth, reverse light orange, stretchy, tacky	More tacky
Lactaria γ	54 mm	Flat, velvety	Entire	Pink rings white sectors	White	Good growth, leathery no odor	Less tacky, white sectors
vellerea $\gamma\beta$	47 mm	Flat, velvety channelled	Undulate	Whitish, pale pink rings	Yellowish	Good growth, pale, tacky, perfumes-odor	Less pigment, more tacky

Mannitol-Diamalt

TABLE XVIII C

Response Variation of Irradiation-Induced Mutants

Species	9 days		30 days		Compared to Norm		
	Diameter	Surface	Margins	Top		Reverse	Texture-Remarks
<i>Lactaria chrysochea</i>	22 mm	Coarse hairy	Slightly undulate	Grey center wide white border	None	Very good growth parchment-like	Larger mat, more grey aerial mycelium
<i>Lactaria</i>	55 mm	Highly raised woolly	Entire	Off white minute droplets	None	Skin-like, flat, pale buff stretchy mat	More pigment in mycelium, less pigment reverse
<i>paludinella</i> γ	55 mm	Highly raised fine densely packed	Entire	White	Light brown	Skin-like, cottony, white	White mycelium, more pigment reverse
<i>Lactaria piperata</i>	30 mm	Highly raised silky	Entire, narrow hyaline border	White	Deep orange-yellow	Good growth, brownish pigment, leathery	Narrow hyaline halo less pigment
<i>Lactaria</i>	25 mm	Raised, rough	Undulate	Brown, narrow white border	None	Good growth, not pruinose rugose, delicate, tacky	More rugose, darker pigment
<i>subdulcis</i> B γ	20 mm	Raised, rough	Undulate	Brown, fine white border	None	Fair growth, rugose, not pruinose, not tacky	Smaller mat, darkest pigment

Mannitol

TABLE XIX

Response-Variation

Species	Diameter	Surface	Margin	Giant Colonies		Reverse	Texture-Remarks
				9 days	30 day		
				Top	Pigmentation		
<i>Lactaria mucida</i>	10 x 15 mm	Raised, cottony	Cottony	White	None	None	Leathery, becoming pigmented, magentared after exposure to light, poor growth
<i>Lactaria paludinea</i>	20 x 23	Raised furry	Cottony	White	Pale orange		Tacky, good growth
<i>Lactaria subdulcis</i> B	17 x 10	Flat smooth	Undulate	Brown center off white	Light brown		Not tacky, very poor growth
<i>Lactaria subdulcis</i> P	6 x 18	Flat smooth	Entire	Whitish	Yellow		Leathery, poor growth yellow pigment diffuses in medium
<i>Lactaria torminosa</i>	50 x 52	Raised, dense silky	Entire	White	Light brown		Leathery, good growth, brown rings, reverse
<i>Lactaria vellerea</i>	50 x 42	Flat, velvety	Irregularly lobed	Rose beige center, white	None		Tacky, wide halo, becoming lavender irregularly reniform

TABLE XIX B

Response Variation of Irradiation-Induced Mutants

Species	Diameter	9 days	Giant Colonies					50 days	Compared to Norm
			Surface	Margin	Top	Pigmentation	Reverse		
<i>Lactaria mucida</i>	10 x 13		Raised spiky	Spiky	White	None	Not rubbery, good growth, much violet to magenta pigment	Mature colony larger more pigment developed	
<i>Lactaria</i>	11 x 15		Flat	Entire	White	None	Skin like, stretchy, good growth, pink, orange reverse	Better growth, strong yellow pigment diffuses into medium	
<i>subtilis Pyl2</i>	12 x 16		Flat	Entire	White	None	Stretchy, pale pink, orange reverse	Much better growth, yellow pigment diffuses into medium	
<i>Lactaria</i>	47 x 50		Raised, dense silky	Entire	White	Light brown	Leathery, stretchy, good growth, reverse brown rings	More stretchy	
<i>terminosa</i>	50 x 57		Raised, dense silky	Entire	White	Pale tan	Tacky, good growth, no rings reverse	More elastic	
<i>Lactaria</i>	30 x 35		Flat velvety	Irregularly lobed	Whitish lavender sector	None	Tacky, whitish pink lavender sectors	Less pigment, irregularly reniform	
<i>vellerea</i>	22 x 35		Flat velvety	Irregularly lobed	Hyaline whitish center	None	Tacky, white, mostly submerged growth	Much less pigment, irregularly reniform	

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