APPRAISAL OF SECONDARY METABOLITES IN *IN VITRO* CULTURES OF *Citrullus colocynthis* (L.) SCHARD.

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ABSTRACT : Plant cell culture systems represent a potential renewable source of valuable medicinal compounds, flavors, fragrances, and colorants, which cannot be produced by microbial cells or chemical synthesis. The present research work was carried out for the analysis of secondary metabolites in callus culture of Citrullus colocynthis (L.) Schard. In vitro grown seedlings were used as explant source. Murashige and Skoog (MS) medium was used as culture medium supplemented with different concentrations and combinations of 2,4-D + Kin mg/L and BAP + NAA mg/L. The combination of BAP 2 mg/L and NAA 3 mg/L was commonly successful in inducing callus in leaf as well as in internode explants while NAA in individual application did not produced good results for callus induction. Laboratory grown seedling parts and the relevant callus cultures raised from different explants were subjected to analysis for secondary metabolites through GC-MS. Among secondary metabolites determined, the common secondary metabolites present in leaf and internode tissues were Toluene, α-sphinosterol, Tetradecane, Pentadecane, Hexadecane, Octadecane, Hentriactone, and 9,12-Octadecadienoicacid[7,7]. Majority of the compounds were detected in quantities less than 1% in seedling tissues as well as in respective calluses. From the present study, it was observed that regardless of the similar compounds found, many metabolites were different in leaf and internode tissues and in respective calluses.

INTRODUCTION

Citrullus, an important genus of the family contains medicinally very important species. *Citrullus colocynthis* (L.) Schard commonly called "Tuma" also known as bitter cucumber, bitter apple, egusi or vine of sodom [1]. It is native of warmer parts of Asia, Syria, Egypt and Morocco for purpose of export. It occurs in the subcontinent including Pakistan and is seen growing wild in the warm and arid sandy tracts [2].

The plant secondary metabolites, important for the human consumption as food and as medicinal compounds used in the pharmaceutical industry require special attention. If the naturally occurring compounds of medicinal importance are produced in *in vitro* cultures, the problem of provision of raw materials for the extraction of bioactive compounds can be solved at door step. Plant parts containing important compounds such as polyphenols, anthocyanins, flavonols and vitamins etc., the main source of natural antioxidants can be cultured on artificial medium as renewable source of required chemical compounds [3]. Different chemical compounds present in the plant material can be detected and analysed. This study was undertaken to analyze and compare secondary metabolites in *in vitro* cultures of *C. colocynthis* and seedling tissues.

MATERIALS AND METHODS

Plant Material and Seed Germination

The seeds procured from market, were decoated and surface sterilized by treating with 2.5% NaOCl (Sodium hypochlorite) solution for aseptic germination. *In vitro* raised seedlings were used as the explant source for callus induction.

Medium Preparation

For medium preparation measured quantities of stock solutions of MS medium [4] were mixed/L for preparation of MS working medium as per requirement. Sucrose was dissolved at the rate of 3.0% (w/v). Calculated quantities of growth regulators (mg/L) for a particular medium were added. The final volume of the medium was made up to the mark by adding double distilled water. pH of the medium was adjusted at 5.8. The medium was jelled with 0.8% agar. The medium was dispensed in appropriate volumes into the culture vessels for sterilization.

Preparation and Incubation of Explant

Leaf, internode and cotyledon from *in vitro* grown seedlings, excised under aseptic conditions, were transferred to the culture vessels containing sterilized media. After culturing the explant, culture vessels containing explants were placed in growth room at temperature of 25˚C ± 3ºC with light period 16 h/ day cycle and 2500 to 3000 lux light intensity.

Subculturing of Callus Culture

To avoid the deficiency of nutrients, callus cultures were transferred aseptically to fresh medium after every 15 days.

Callus Index

Callus index (CI) was calculated by the following formula [5].

CI=100n x G/N (n = Number of explants initiating callus, G=Visual callus rating of initiated explants, N = Total number of explants planted)

ANALYSIS OF SECONDARY METABOLITES BY GC-MS

The calli biomass was crushed (if compact in nature) and ground by using a porcelain pestle mortar. The homogenous slurry was diluted and semisolid mass and solvent mixture was separated by filtration. The solvent mixture was distilled in water bath in a round bottom flask to obtain essential oil. The combined solvent extract was dried over anhydrous sodium sulphate (Na2SO4) and the solvent was distilled off, leaving the essential oil as a residue in the flask. The essential oil was subjected to GC-MS.

For GC-MS analysis, sample of steam distilled oil was injected and run under the following conditions: Capillaray Column of HP-5ms 5% nonpolar, column at Inlet temperature 200°C and detection Temperature 250°C. Helium gas used as Carrier Gas with flow rate1.0 ml/min. Column Oven Programing, 50-230min @ 5ºC/m.

RESULTS

Seed germination

During seed germination, the seeds were subjected to different soaking periods such as 1, 2, 3, 4 and 12 hours. No

germination was observed in seeds with soaking time periods from 1 to 4 hours while seed germination was observed only in seeds soaked for 12 hours. Five seeds per petriplate were employed, while amount of water used to moist the cotton pad per petriplate ranged from 10 to 25ml with the interval of 5ml. No germination was observed in petriplates having cotton pads soaked with 15, 20 and 25ml of water. Germination was observed in petriplates moistened with 10ml of water.

Callus induction

Different parameters studied were callus initiation, callus induction %age, duration of callus induction, callus index, callus colour and callus texture. Callogenesis was observed in leaf and internode explants of *C. colocynthis* while cotyledon failed to respond. Time duration for callus initiation from leaf of *C. colocynthis* was 3 to 8 days. Callus formation initiated at the margins. In MN5 callus induction was 33% where as callus index was 33 and embryogenic green callus was formed from leaf explants (Table 1). In MBN2,3 (Table 1) callus induction was 100% where as callus index was 300 and embryogenic brownish green callus was formed from leaf as shown in Fig 2. Good results for callus induction were observed in MBN2,3. Time duration for callus induction from internode of *C. colocynthis* was also 3-8 days. In MN5 callus induction was 33% where as callus index was 33 and embryogenic green callus was formed from internode explants (Table 1). In MBN2,3 callus induction was 66% where as callus index was 200 and embryogenic light green callus was induced on internode(Table1).

Table 1: Effect of different concentrations of BAP + NAA mg/L for the callus induction from leaf of *C. colocynthis*

Explant Type	Medium Code	¶PGRs (BAP/ NAA mg/L)	No of explant producing callus	* CI %age	** VCR	*** CI	Initiation Duration (Days)	Character -istics of Callus
Leaf	MN5	-/5	1	33	1	33	8	Green, ⇑E
-	MBN2,3	2/3	3	100	4	300	4	Brownish green, E
Internode	MN5	-/5	1	33	1	33	8	Green, E
-	MBN2,3	2/3	2	66	4	200	3	Light green, E

No of replicates = 3 ¶Plant Growth Regulators *Callus Induction %age, **Visual Callus Rating: 1=25%, 2=50%, 3=75%, 4=100%, ***Callus Index, ⇑Embryogenic

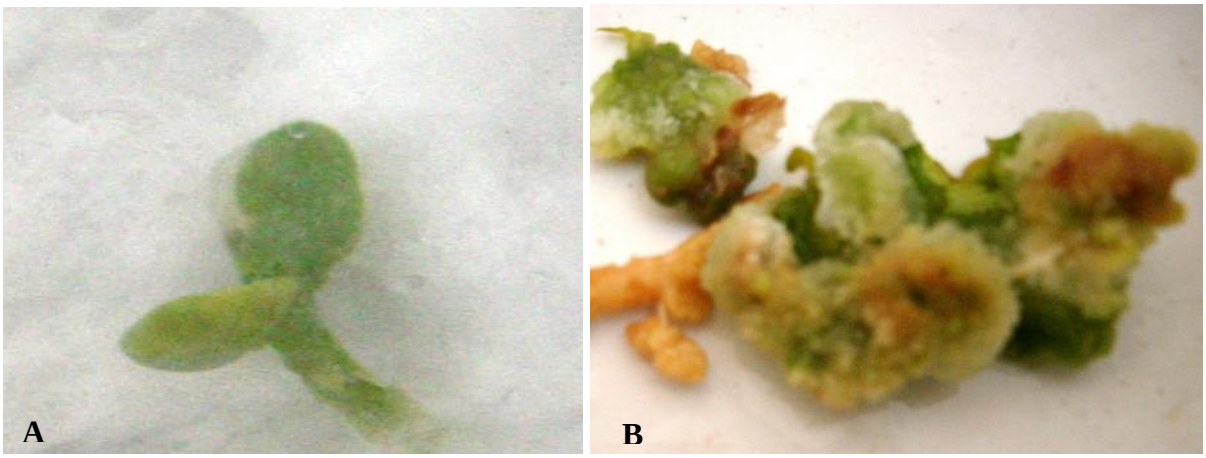

Fig 1: *In vitro* grown seedling and callus induction in *C. colocynthis*. **A)** 30 days old seedlings of *C. colocynthis* (2x) **B)** 19 days old callus induction on leaf of in medium in BAP 3mg/L + NAA 2mg/L (2.5x)

GC-MS ANALYSIS OF *IN VITRO* GROWN SEEDLING AND CALLUS CULTURES

Different compounds, as secondary metabolites from leaf and leaf callus cultures under the influence of different PGRs, were detected in different quantities from different sources, (Table 2). Compounds more than 10% were Toluene (63.515), Benzene1,3-dimethyl (12.589), Undecane (52.29) and Aniline, N-methyl (13.964), compounds from 1 to 10% were Ethylbenzene (2.771) and *o*-Xylene (3.097) whereas compounds less than 1% were Nonane (0.426), Nonane (0.168), 2-Propenoic acid (0.372), Undecane (0.082), Dodecane (0.074), Tridecane (0.077), Tetradecane (0.069), Pentadecane (0.045), Hexadecane (0.054) and Octadecane (0.031) detected in callus cultures under the influence of MBN2,3 medium. The same trend was found in internode and internode derived cultures (Table 3), the compounds detected are can be classified into three categories according their percentage of occurrence.

Table 2: Percentage composition of secondary metabolites from seedling-leaf and leaf callus under different concentrations of PGRs.

Sr. #	Names of compounds detected through GC-MS	Retention Time (min) /Average %age of compounds in leaf tissue	Retention Time (min)/Average %age of compounds in leaf callus (MBN0,5)	Retention Time (min)/Average %age of compounds in leaf callus (MBN2,3)
1	Toluene	3.89/1.692	3.59/20.4	3.74/63.51
2	Nonane	4.82/1.184	4.87/.127	4.12/0.426
3	Benzene 1,3-dimethyl	5.99/0.649	-	6.43/12.58
4	Ethylbenzene	6.10/0.237	6.09/3.93	6.09/2.77
5	*o*-Xylene	-	7.22/3.081	7.58/3.097
6	2-Propanoic acid, butyl ester	7.89/0.424	7.76/0.619	7.75/0.372
7	3-Cyclohexene-1-01,4-methyl-1-[1-methylethyl]	-	10/2.111	-
8	α-Sphinosterol	12.03/0.250	-	-
9	Undecane	12.25/15.348	12.04/52.29	12.24/0.82
10	Dodecane	13.65/0.120	13.65/0.120	13.65/0.074
11	Tridecane	-	14.60/0.118	13.60/0.077
12	Aniline, N-methyle	-	-	14/13.96
13	2,6octadiene,-1-01,3,7-dimethyl-acetate,-	14.99/0.858	-	-
14	Tetradecane	15.36/0.173	15.36/0.77	15.36/0.069
15	Pentadecane	16.02/1.692	16.02/0.079	16.02/0.045
16	Hexadecanes	16.61/0.240	16.61/0.043	16.61/0.054
17	Heptadecane	-	17.17/0.053	-
18	Octadecane	17.69/0.984	17.69/0.01	17.69/0.031
19	Hentriactone	19.89/0.280	-	-
20	1,2 Benzenedicarboxylic acid,diisoctylether	-	-	21.43/1.667
21	9,12-Octadecadienoicacid[7,7]	22.21/70.893	-	-
22	n-Hexadecanoic acid	26.02/0.187	-	-

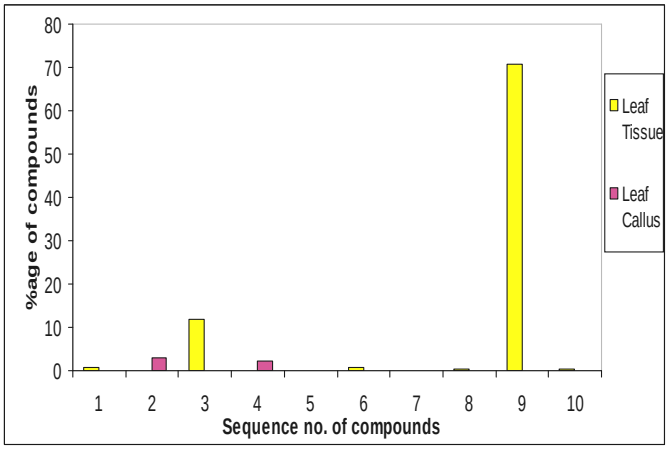

Fig. 2: Secondary metabolites found different in seedling-leaf tissue and leaf callus under the influence of NAA 5 mg/L.

Sequence of compounds shown in the graph:

(1) Benzene1,3-dimethyl (2) o-Xylene (3) α-sphinosterol (4) 3-Cyclohexene-1-01,4-methyl-1-[1-methylethyl] (5) Tridecane (6) 2,6-octadiene,-1-01,3,7-dimethyl-acetate (7) Heptadecane (8) Hentriactone (9) 9,12-Octadecadienoic acid [7,7] (10) n-Hexadecanoic acid.

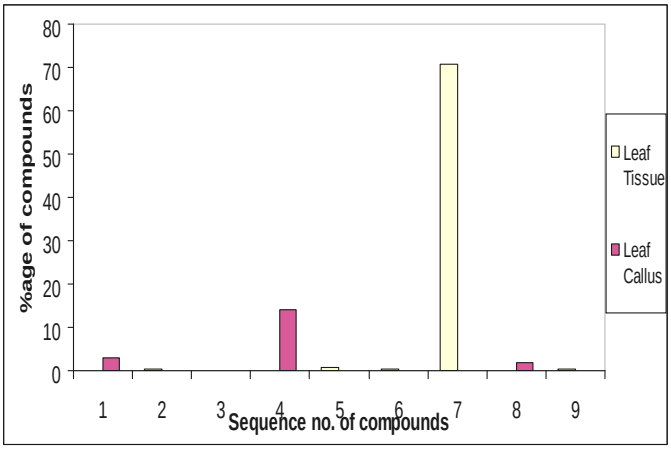

Fig. 3: Secondary metabolites found different in seedling-leaf tissue and leaf callus under the influence of BAP 2 mg/L + NAA 3 mg/L.

Sequence of compounds shown in the graph:

(1) o-Xylene (2) α-sphinosterol (3) Tridecane (4) Aniline, N-methyl (5) 2,6octadiene,-1-01,3,7-dimethyl-acetate (6) Hentriactone (7) 1,2 Benzenedicarboxylic acid,diisoctylether (8) 9,12-Octadecadienoicacid[7,7] (9) n-Hexadecanoic acid.

Table 3: Percentage composition of secondary metabolites from seedling-internode and internode callus under different concentrations of PGRs.

Sr. #	Names of compounds detected through GC-MS	Retention Time (min) /Average %age of compounds in Internode	Retention Time (min)/Average %age of compounds in internode callus (MBN0,5)	Retention Time (min)/Average %age of compounds in internode callus (MBN2,3)
1	Toluene	3.92/0.162	3.39/13.96	3.59/31.85
2	Nonane	-	4.86/0.426	4.86/0.127
3	Ethyle benzene	-	6.09/14.707	-
4	Benzene 1,3-dimethyl	-	6.42/3.93	-
5	β-Phellandrene	6.73/0.495	-	-
6	o-Xylene	7.53/5.434	7.58/2.77	7.52/1457
7	2-Propanoic acid, butyl ester	-	7.76/1.866	7.66/6.19
8	Iso-orientin	870/0.237	-	-
9	6-C-p-hydroxybenzoylvitexin	9.26/0.424	-	-
10	8-C-p-hydroxybenzoylvitexin	9.68/0.437	-	-
11	Citrullol	11.19/0.583	11.99/23.097	-
12	Undecane	-	12.25/0.372	12.25/0.111
13	Elatericin B	11.47/1.776	-	-
14	Dodecane	-	13.65/0.082	13.65/ 0.12
15	α-sphinosterol	12.03/0.858	-	-
16	Tridecane	-	14.60/0.194	14.60/0.118
17	Tetradecane	15.420.341	15.36/0.077	15.36/0.077
18	Pentadecane	16.13/1.184	16.02/0.069	16.02/0.069
19	αElaterin	-	14.10/44.29	-
20	Hexadecane	16.61/0.108	16.61/0.045	16.61/0.043
21	Octadecane	17.69/2.623	-	-
22	Heptadecane	-	17.17/0.054	-
23	Hentriactone	19.89/0.240	-	-
24	9,12-Octadecadienoicacid[7,7]	22.01/1.221	-	-
25	Elaterin	25.99/0.280	-	-
26	n-Hexadecanoic acid	26.42/82.585	-	-

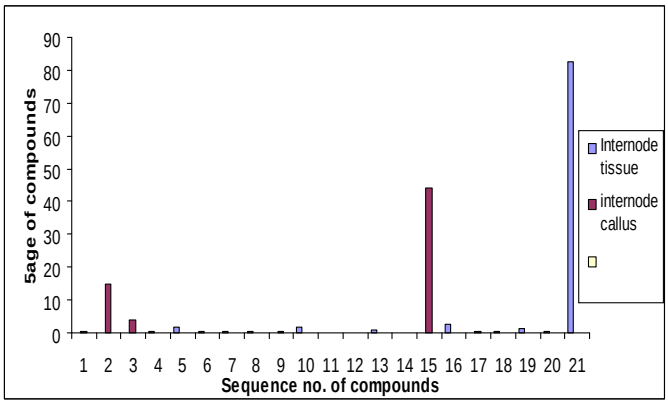

Fig. 4: Secondary metabolites found different in seedling-internode tissue and internode callus under the influence of NAA 5 mg/L

Sequence of compounds shown in the graph:

(1) Nonane (2) Ethylbenzene (3) Benzene 1,3-dimethyl (4) β-Phellandrene (5) 2-Propanoic acid, butyl ester (6) Iso-orientin (7) 6-C-p-ydroxybenzoylvitexin (8) 8-C-p-hydroxy -benzoylvitexin (9) Citrullol (10) Undecane (11) Elatericin B (12) Dodecane (13) α-sphinosterol (14) Tridecane (15) α-Elaterin (16) Octadecane (17) Heptadecane (18) Hentriactone (19) 9,12-Octadecadi enoicacid[7,7] (20) Elatrin (21) n-Hexadecanoic acid

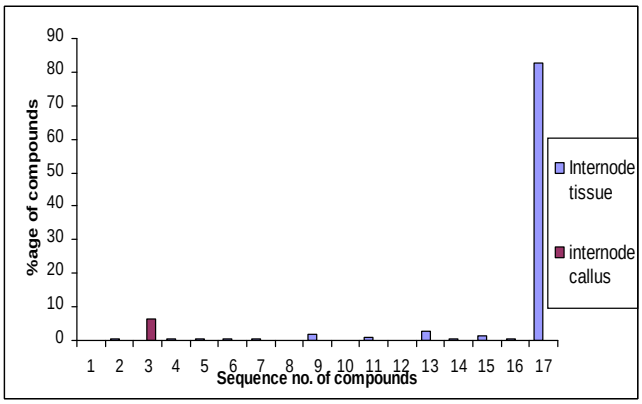

Fig. 5: Secondary metabolites found different in seedling-internode and internode callus under the influence of BAP 2 + NAA 3 mg/L

Sequence of compounds shown in the graph:

(1) Nonane (2) β-Phellandrene (3) 2-Propenoic acid, butyl ester (4) Iso-orientin (5) 6-C-p-hydroxybenzoylvitexin (6) 8-C-p-hydroxy -benzoylvitexin (7) Citrullol (8) Undecane (9) Elatericin B (10) Dodecane (11) α-sphinosterol (12) Tridecane (13) Octadecane (14) Hentriactone (15) 9,12-Octadecadienoicacid[7,7] (16) Elaterin (17) n-Hexadecanoic acid.

DISCUSSION

Effect of different combinations of PGRs on *in vitro* cultures is well pronounced. In application of PGRs to raise *in vitro* cultures, the combined effect of different concentrations of BAP/NAA was prominent such as medium code MBN2,3 containing BAP/NAA in a ratio of 2:3 mg/L, showed quick response to initiate embryogenic callus in shorter time duration with callus index of 300 from leaf explant (Table 1). The same was observed for internode explant with callus index of 200. The callus from leaf explant was brownish green and yellowish green from internode. The similar results have been described by many workers [6][7], they found that the highest percentage of callus cultures was derived from explants grown on BAP/NAA combinations than BAP/NAA alone. In MN,5 (NAA 5mg/L) callus induction recorded was 33% with callus index of 33. The embryogenic green callus was generated from both leaf and internode explant. These results were supported by Attard and Spiteri who demonstrated that media containing 5 mg/L NAA did not support to induce high percentage of callus in *Ecballium elaterium* [8].

Leaves, roots, flowers and fruits consist of complex cellular tissues containing compartmentalized phytochemicals. Cell, callus and organ cultures of plants are capable of producing most types of secondary compounds, including phenylpropanoids, polyketides, terpenoids, alkaloids, and carbohydrates, and offer excellent materials for biosynthetic studies because they consist of populations of actively growing cells [9]. The analysis of *in vitro* cultures through GC-MS revealed the presence of flavonoids, steroids, alkaloids and saponins. Presence of flavonoids has been reported from many plant species like *Lycium barbarum* [10], *Passia palmer* [11], *Cassia angustifolia* [12], *Jatropha curcus* L. [13]. The presence of compounds such as iso-vitexin, iso-orientin and iso-orientin 3'-methyl ether, 8-C-p-hydroxybenzoylisovitexin 4'-O-glucoside was also detected. As described by Matooq *et. al.,* they identified six flavone C-glycosides [14]. Similary Hatam *et al.,* also reported the presence of glycosides 2-O-β-d-glucopyranosyl in *C. colocynthis* [15]. The tables 3 and 4 show the similarities and differences as regards the presence of secondary metabolites in seedling leaf and internode tissue and leaf and internode calluses respectively. Many metabolites from seedling tissue and its respective callus were different. The differences in secondary metabolites in leaf and leaf callus and internode and internode callus are summarized in Figs. 2 to 5. Mulabagal and Tsay, observed differences in content of the induced callus among the six source organs [3]. Analysis by HPLC revealed that both stem-node and microtuber derived suspension cells contained diosgenin. The content of microtuber derived cell suspension culture contained 3.2% diosgenin per gram dry-weight, where as, the stem-node derived cultures contained only 0.3%. Callus from microtuber showed yellowish color and embryogenic in appearance and contained the highest diosgenin content (3.3-3.5%). The amount of diosgenin obtained from tuber derived cell suspension was high and comparable with that found in the intact tuber [16]. Gamborg *et al.,* also suggested that the metabolites produced in seedling tissue may be different from the metabolites produced in respective callus [17].

The kinds and concentrations of PGRs also influence the production of secondary metabolites. According to Mulabagal and Tsay and Asrafi *et al.,* for establishing a rapidly growing and finely dispersed cell suspension culture, the best medium composition was ½ strength of MS medium supplemented with 1 mg/L 2,4-D, 0.1 mg/L kinetin, and 3% sucrose. All the cultures were routinely sub cultured at an interval of 14 days [3][18]. It was found that MS basal media was the best among the media tested on imperatorin production. When influence of auxins on imperatorin production was investigated, deletion of auxins from the medium was beneficial to imperatorin production and addition of BA (0.5-1 mg/L) promoted synthesis of imperatorin in suspension cells. As was observed in this study that callus cultures from leaf explant raised on MS medium supplemented with NAA at a concentration of 5 mg/L were lacking the compounds like Benzene 1,3-dimethyl and Aniline, N-methyl (Table 2), but on reducing the amount of auxin and addition of BAP (BAP/NAA, 2/3 mg/L) resulted in the production of the said compounds. The concentration of these compounds increased manifold in the callus cultures than the leaf tissue. The results were found vice versa in the internode derived callus cultures where the same combination of PGRs supplemented in the same way retarded the production of some compounds which were being produced in the internode callus cultures influenced by NAA (5 mg/L). From the comparative analysis of secondary metabolites, it is concluded that most of the secondary metabolites from seedling tissue were present in their respective calluses. From the present study, it was also observed that many of the metabolites from seedling tissues and their respective calluses can vary under the influence of different combinations PGRs.

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