TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2015) 39: 479-484

doi:10.3906/biy-1406-85

Evaluation of the phytoremediation capacity of *Lemna minor* L. in crude oil spiked cultures

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Received: 29.06.2014 • **Accepted/Published Online:** 19.02.2015 • **Printed:** 15.06.2015

Abstract: Phytoremediation of freshwater contaminated with crude oil is a technology that can restore damaged freshwater areas. *Lemna minor* is a small vascular plant that reproduces rapidly, is sensitive to a wide variety of pollutants, and is easy to culture. This study aimed to evaluate the phytoremediation capacity of *L. minor* in crude oil spiked cultures. Cultivation was carried out for 7 days in a greenhouse with a natural photoperiod and in nutrient solutions containing 0.5%, 1%, 2%, and 3% crude oil. Extracts were analyzed using GC/MS and synchronous UV fluorescence spectroscopy. After a week of cultivation, the fresh weight of plants in the control medium increased by 117%. The presence of crude oil up to 0.5% v/v reduced growth as much as 50% relative to the control plants. C_{17}/Pr and C_{18}/Ph ratios decreased especially in the presence of 0.5% to 2% v/v crude oil in the growth media. In 0.5% oil concentrations, both unplanted control samples and plant samples contained no 1–4 ring polycyclic aromatic hydrocarbons. However, at oil applications of 0.5% and 1%, the plant samples contained 5 ring polycyclic aromatic hydrocarbons; their intensity was approximately two times lower than that of the unplanted control samples. It can be concluded that the biodegradation potential of *L. minor* strongly depends on the concentration of crude oil contaminants. Finally, the phytoremediative capacity of *L. minor* is only suitable for cleaning of freshwater resources containing small amounts of oil contaminants.

Key words: Crude oil, freshwater, pollution, growth inhibition, isoprenoids, polycyclic aromatic hydrocarbons

1. Introduction

The release of a large number of pollutants, especially organics such as crude oils, polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, and pesticides, into the environment has resulted in the contamination of soil and surface and ground waters (Dordio and Carvalhio, 2013). These compounds diffuse into aquatic environments through different means such as industrial discharges, petroleum spills, combustion of fosil fuels, automobile exhausts, urban runoff, and atmospheric fall-out (Olajire et al., 2005). Water contaminated with hydrocarbons is common in the oil-producing and industrialized countries of the world and is a considerable threat to the environment and to human health (Al-Baldawi et al., 2015). Oil spills also occur in freshwater environments through accidental discharge during oil exploration and transportation. Contamination with crude oil and PAHs can have adverse effects on organisms and water quality (Venosa et al., 2002; Lin and Mendelssohn, 2009).

Conventional oil spill clean-up methods include various physical (booming, skimming, wiping, mechanical removal, washing, and tilling) and chemical (dispersants, demulsifiers, solidifiers, and surface film chemicals) processes (Ndimele and Ndimele, 2013). However, traditional oil spill clean-up activities may do more damage to the aquatic ecosystem than the oil spill itself (Lin and Mendelssohn, 1998). Therefore, it is necessary to find alternative methods to restore the oil contaminated freshwater areas. Bioremediation is a promising approach for cleaning up environmental contaminants with the use of organisms (Kigigha and Underwood, 2009; Pandey and Fulekar, 2012). Phytoremediation uses plants and their associated microorganisms to restore water contaminated with hydrocarbons, and is more environmentally friendly

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than the conventional mechanical clean-up methods (Barrutia et al., 2011; Liu et al., 2011). Aquatic plants and associated microbial communities may contribute to the removal and biotransformation of xenobiotic compounds from contaminated water and sediments (Rice et al., 1997). Duckweeds (Lemnaceae) have gained broad applications in ecotoxicological research as model organisms (Suresh Kumar and Han, 2010). They grow quickly and reproduce faster than other vascular plants. There are several studies on the effects of organic contaminants on these plants (Park et al., 2012; Coronado-Posada et al., 2013; Zezulka et al., 2013). Many studies have been conducted on the cleaning of crude oil polluted soils with phytoremediation using different plant species (Wu et al., 2009; Moubasher et al., 2015; Xiao et al., 2015). However, the literature on the use of freshwater plants for phytoremediation of crude oil is limited.

The objective of this study was to shed light on the removal capacity of *Lemna minor* grown in a culture medium spiked with different concentration of crude oil through the evaluation of PAHs uptake.

2. Materials and methods

2.1. Culture preparation and application of crude oil

L. minor was originally collected from the Botanical Garden of the Faculty of Science at İstanbul University. The plants were grown in a Hoagland nutrient solution containing 136 mg L^{-1} KH_2PO_4, 236 mg L^{-1} $Ca(NO_3)_2{\cdot}4H_2O$, 101.1 mg L^{-1} KNO_3, 246 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 2.86 mg L^{-1} H_3BO_3, 1.82 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0.22 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.09 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$, 0.09 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 4.84 mg L^{-1} $FeCl_3{\cdot}6H_2O$, and 15 mg L^{-1} Na_2EDTA. The pH value of the nutrient solution was adjusted to 6.0. Plants were grown at 25–28/19–21 °C day/night temperature, and 60%–65% humidity under greenhouse conditions.

Batman crude oil from the Batman Refinery (Batman, Turkey) was used. The effects of different amounts of Batman crude oil (0.5%, 1%, 2%, and 3% by volume) on the growth of *Lemna minor* after a 7 day exposure were investigated. A Hoagland nutrient solution without plants, containing different oil concentrations, was used as a negative control (unplanted control). A day after the application of crude oil, 6 g of the plant was transferred into each pot (2 L). After 7 days of exposure, the plants were weighed and stored at –80 °C until analysis. The effects of crude oil on the growth of *L. minor* were measured by comparing the fresh weight of the plants before and after the application of different concentrations of crude oil. Eighty-milliliter aliquots from the unplanted control media were taken and stored at 4 °C until extraction.

L. minor relative growth rate (RGR) (g g^{-1} day^{-1}) was calculated by the following formula:

RGR = (ln W2 – ln W1)/t, where W1 and W2 are the initial and final FW (g), and t is the experimental time (days) (Jampeetong and Brix, 2009).

2.2. Extraction and clean-up procedures

The unplanted control media samples and plant samples (3 g) were extracted according to the USEPA Method 3510 and a slightly modified USEPA method 3541. All extracts were fractioned with adsorption chromatography with a Florisil column and topped with sodium sulfate anhydrous. The extracts (0.5–1 mL) were carefully added to the columns and two fractions were collected: (1) 10 mL of hexane (aliphatic hydrocarbons); and (2) 10 mL of 1:1 dichloromethane:hexane (aromatic compounds) (modified EPA 3600C). All of the extracts were concentrated by a gentle N_2 blow-down to ~ 100 µL.

2.3. Spectrofluorometric analysis of PAHs

UV fluorescence in synchronous excitation-emission technique has been a promising tool for determining the polyaromatic structure of a compound since its development by Lloyd (1971). Briefly, this method relies on the fact that compounds in synchronous spectra exhibit their maximum emissions at particular wavelengths, depending on the number of fused aromatic rings in their chemical structures (Kister et al., 1996).

The unplanted control media samples and the plant samples were analyzed by synchronous UV fluorescence spectroscopy (SUVF, Jasco-6300, Shimadzu). For quantitative characterization, the excitation wavelength was scanned from 220 to 700 nm. The $\Delta\lambda$ interval between λ_{ex} and λ_{em} was constant and equal to 23 nm (Lloyd, 1971). Each sample was analyzed three times in order to evaluate the reproducibility of the method. Quartz cuvettes (1 cm in length) were used for the measurements.

2.4. GC/MS analysis

The extracts were analyzed by gas chromatography/mass spectrometry (GC/MS) (PerkinElmer Thermo DSQ Turbo MSD) with an HP-5MS capillary column (30 m × 0.25 mm i.d.: 0.25 µm of film thickness). The GC oven temperature program was maintained at 50 °C for 1 min, increased from 50 to 320 °C at 10 °C/ min, and then held at 320 °C for 5 min. The carrier gas was helium at a constant flow rate of 1.0 mL/min. The front inlet temperature was 280 °C and the injector was set to splitless injection. The MS temperature program for the transfer line was 220 °C. The MS was operated in EI mode (70 eV), scanning from 50 to 600 amu. The library search was carried out using Wiley and NIST GC/MS libraries. The GC/MS was calibrated for the aliphatic hydrocarbons (n-alkanes calibration mixture purchased from Dr Ehrenstorfer, Germany) from nC_{10} to nC_{35} by using the internal standard calibration procedure described in US EPA Method 8015. Retention indices and the mass spectra of the primary ion were detected using GC/MS analysis. Isoprenoid ratios (C_{17}/Pr, C_{18}/Ph, and Pr/

Ph) were detected using the peak areas and the secondary intensity measures. All of the solvents were of HPLC grade.

2.5. Statistical analysis

All the experimental data were obtained in 3 replicates. The experimental results are expressed as mean ± standard deviation (SD) of triplicate measurements and analyzed by GraphPad Prism version 5.2 for Windows (GraphPad Software, USA). Significant differences among the means were determined by the post-hoc Bonferroni test and Tukey's multiple comparison test.

3. Results

Even though the growth of *Lemna minor* plants was significantly hindered by the presence of crude oil in the growth media, the plants were able to survive in all oil applications. At the end of the 7-day experimental period, the fresh weight of the plants in the control medium (0%) increased by 117% relative to their initial fresh weight (Figure 1A). As Figure 1A shows, in the presence of oil concentrations of 0.5%, 1%, 2%, and 3%, the plant growth was decreased in comparison with the control by 50%, 58%, 68%, and 89%, respectively. Relative growth rates of *L. minor* decreased in the presence of Batman crude oil in a concentration-dependent manner (Figure 1B). Furthermore, the relative growth rate of plants in 0.5% crude oil concentration medium did not differ from the 1% crude oil application.

The total ion chromatogram of Batman crude oil can be seen in Figure 2. In order to determine the petroleum hydrocarbon degradation capacity of *L. minor*, isoprenoid ratios for each oil application are given in the Table. The C_{17}/pristane (C_{17}/Pr), C_{18}/phytane (C_{18}/Ph), and pristane/phytane (Pr/Ph) ratios were 3.45, 2.33, and 0.83 respectively, for Batman crude oil (Table). Furthermore, the C_{17}/Pr ratio

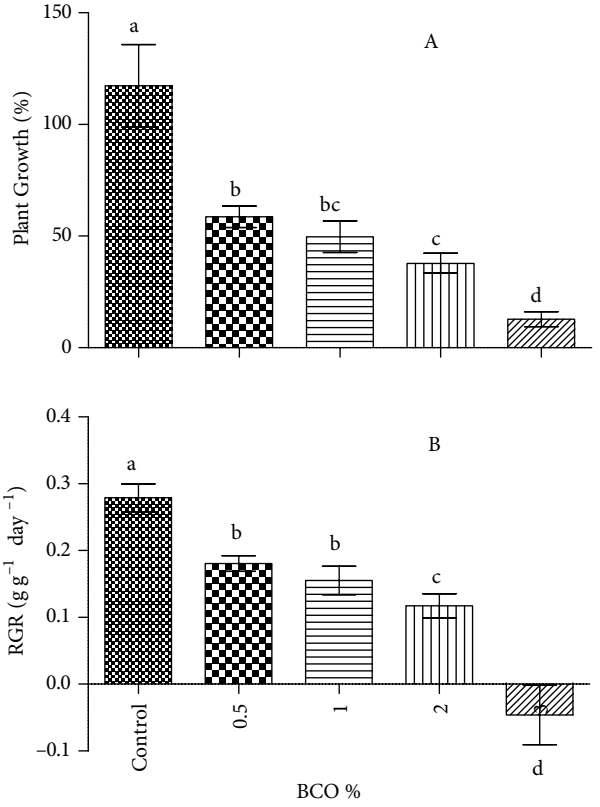

Figure 1. The effect of Batman crude oil (BCO) on the growth of *L. minor* on day 7 of crude oil application. **A.** Relative growth rates of *L. minor* treated with BCO; **B.** Standard deviations are presented by error bars. Significant differences were determined by Tukey's multiple comparison test ($P < 0.05$) and are indicated by different letters.

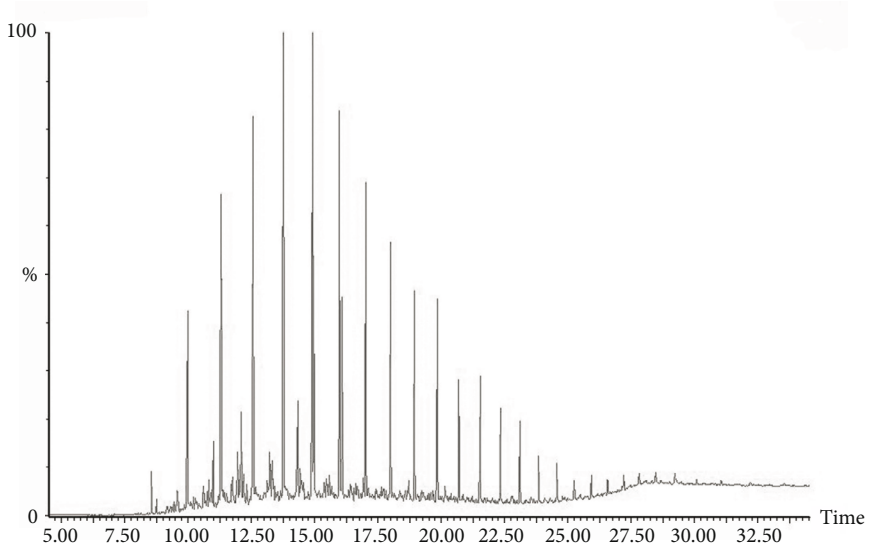

Figure 2. Total ion chromatogram of Batman crude oil at the end of day 7.

Table. Isoprenoid ratios (C_{17}/Pr, C_{18}/Ph, and Pr/Ph) obtained from the Batman crude oil (BCO) applications, unplanted media samples, and the plant samples at concentrations of 0.5%–3% at the end of day 7 of applications. (Value ± SD) '(*), (**), (***)' indicate significant difference from the control: $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively. Significant differences determined by the post-hoc Bonferroni test.

Application concentration (%)	Experiment series	Isoprenoid ratios		
		C_{17}/Pr	C_{18}/Ph	Pr/Ph
	BCO	3.45 ± 0.32	2.33 ± 0.27	0.83 ± 0.10
0.5	Control	2.51 ± 0.19	1.95 ± 0.32	0.22 ± 0.04
	L. minor	1.58 ± 0.20$^{(***)}$	1.16 ± 0.15$^{(***)}$	0.19 ± 0.02
1	Control	2.81 ± 0.19	1.82 ± 0.15	0.40 ± 0.09
	L. minor	2.11 ± 0.11$^{(***)}$	1.52 ± 0.04$^{(**)}$	0.35 ± 0.03
2	Control	3.02 ± 0.07	2.25 ± 0.07	0.75 ± 0.02
	L. minor	2.44 ± 0.18$^{(***)}$	1.62 ± 0.03$^{(***)}$	0.67 ± 0.07$^{(*)}$
3	Control	2.93 ± 0.08	2.23 ± 0.13	0.81 ± 0.02
	L. minor	2.71 ± 0.22$^{(*)}$	1.98 ± 0.14$^{(**)}$	0.77 ± 0.02

was 2.51 for the unplanted control media samples at 0.5% oil concentration; this ratio was much lower (1.58) for the plant samples. C_{17}/Pr and C_{18}/Ph ratios showed a decrease, especially at the range of 0.5% to 2% crude oil concentrations, relative to the unplanted control media samples. However, Pr/Ph values obtained from all oil applications were similar to the ones obtained from the unplanted control media samples. According to the data obtained from the unplanted control media samples, the biodegradation percentages of C_{17}/Pr and C_{18}/Ph of the plant samples at 0.5% oil concentrations can be calculated as 37% and 41%, respectively (Figure 3).

SUVF values obtained from the unplanted control media samples and the plant samples are shown in Figure 4. In 0.5% oil concentrations, both the unplanted control media samples and the plant samples contained no 1–4 ring PAHs. Biodegradation of PAHs should: 1) decrease with increasing molecule size; and 2) decrease within a homologous series with increasing number of alkylations (Kennicutt, 1988). At oil applications of 0.5% and 1%, the intensity of the 5-ring PAHs absorption peak was lower for the plant samples than it was for the unplanted control media samples. However, the absorption peak size for PAHs with 6 or more rings was at a similar intensity for both the unplanted control media samples and the plant samples (Figure 4).

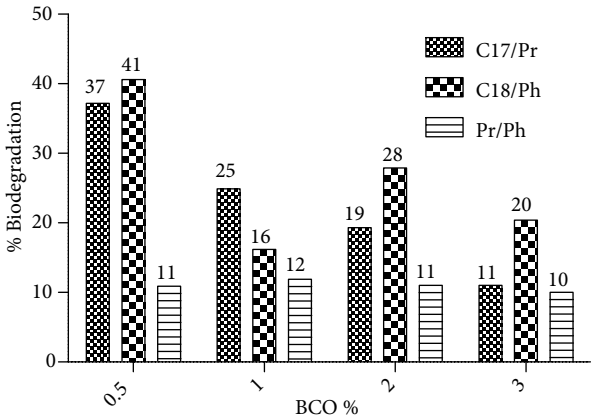

Figure 3. Biodegradation percentages of isoprenoids (C_{17}/Pr, C_{18}/Ph, and Pr/Ph) in the plant samples compared with the unplanted media samples in the presence of Batman crude oil concentrations in the range of 0.5%–3% at the end of day 7 of applications.

4. Discussion

Merkl et al. (2004) have analyzed in detail the adverse effects of crude oil on plant growth. Sharifi et al. (2007) suggested that the severity of the inhibitory effect of crude oil on plant growth in contaminated soils was strictly dependent on the amount of pollutant. In this study, plant growth was inhibited in a dose dependent manner with increasing percentages of crude oil in the media. We observed that the presence of crude oil at 0.5% to 1% in the medium stunted plant growth by over 50% as compared with the control. In media with more than 2% crude oil concentrations plant growth was almost completely inhibited. The RGR reflects the health of plants during the experimental period. The RGRs were 0.26, 0.18, and 0.15 g g^{-1} $day^{-1}$for the control, the 0.5%, and the 1% crude oil application, respectively. Our RGR results also indicate that crude oil concentrations (0.5%–3%) had a negative effect on plant growth by reducing RGR values. In this study,

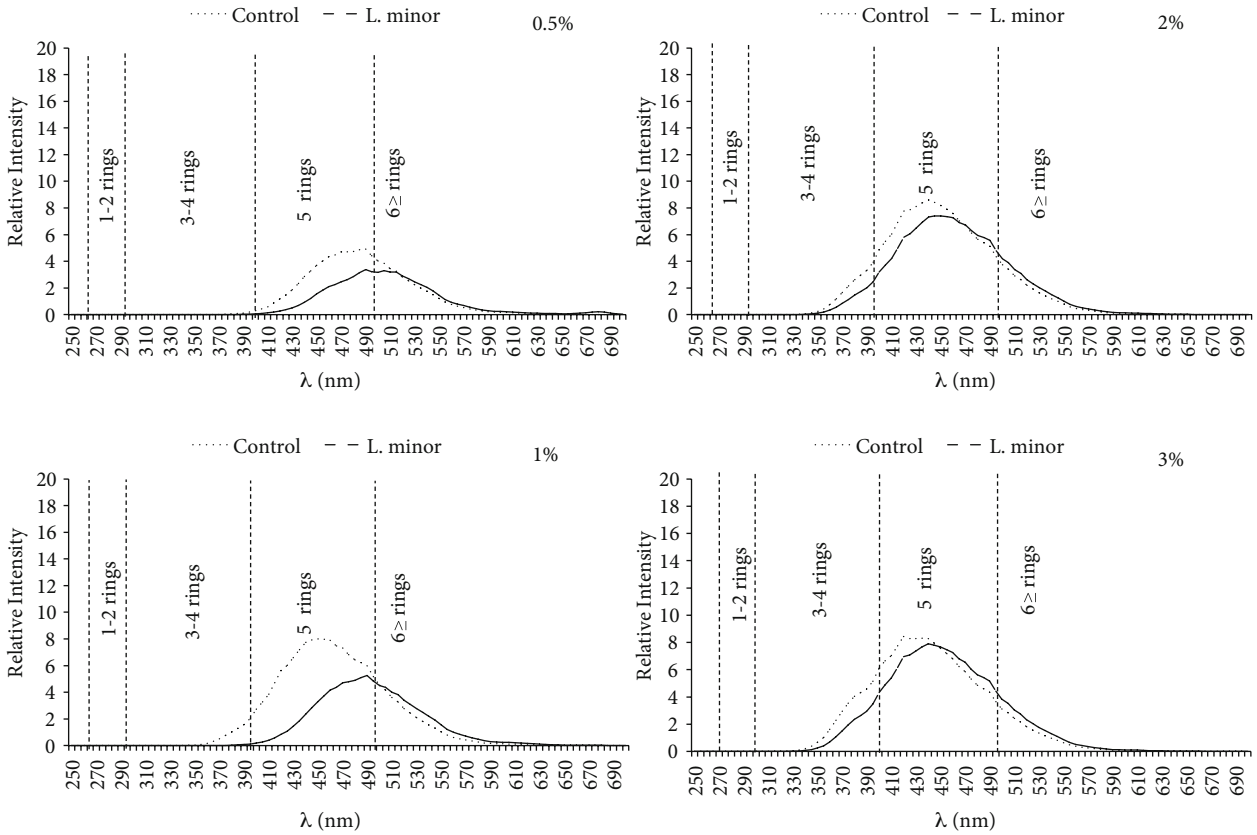

Figure 4. Synchronous excitation/emission (nm) fluorescence spectra of aromatic hydrocarbon fractions of the unplanted media samples and the plant samples from media containing 0.5%–3% Batman crude oil by volume.

RGR values obtained from the different crude oil concentrations were similar to the results obtained by Al-Baldawi et al. (2015). The hindering effect of oil on plant growth may be a result of the toxic effects of low molecular weight hydrocarbons in petroleum (Zand et al., 2010).

Pristane and phytane are usually used as biomarkers for early stages of bioremediation. Pristane and phytane are subject to the same physical and chemical removal mechanisms as their corresponding straight-chain alkanes. Therefore, C_{17}/pristane and C_{18}/phytane ratios have been traditionally used to gauge the extent of biodegradation (Zhu et al., 2001). Evaluating *L. minor* plant growth and its effects on biodegradation, we can say that *L. minor* was effective in up to 2% crude oil concentrations (Figure 1; Table).

The fluorescence of crude oil stems from the presence of PAHs, which are formed by combinations of one or more highly fluorescent benzene rings (Abbas, 2006). According to the number of aromatic rings, priority PAH compounds are divided into several groups, representing two-, three-, four-, five-, and more-ringed PAHs (Law, 1981). In the current study, in both unplanted control media samples and plant samples 1–2 ring PAHs were not encountered. It appears that the more volatile PAHs are rapidly lost in growth media through evaporation and photooxidation (Figure 4). For the applied oil concentrations of 0.5%–3% v/v, the intensity of 5 ring PAHs in plant samples was lower than the intensities obtained from unplanted control media samples, especially at the 0.5% and 1% oil applications. Contaminant removal mechanisms involved in phytoremediation are complex and not limited only to the direct metabolism of contaminants by plants. The PAHs are mainly localized in the roots; the plant plays a significant role as a stabilizer and extractor (Reynoso-Cuevas et al., 2011). Thus, for the 5 ring PAHs, probably the root of *L. minor* plays the role of an extracting device because we found the concentration of 5 ring PAHs to be approximately two times lower at the 0.5% and 1% oil application samples as compared with the controls.

In conclusion, the present findings suggest that crude oil adversely influences the growth of *L. minor.* Furthermore, it can be suggested that the phytoremediative capacity of *L. minor* is only suitable for cleaning of freshwater resources containing small amounts of oil contaminants.

Acknowledgment
This work was supported by the Scientific and Technological Research Council of Turkey, Project number, 105G079.

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