Research Square

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Activation of MAPK signaling pathway during nitrogen-deficiency responses in Ulva Prolifera

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Abstract

Background: Ulva prolifera is one of the main seaweeds (or macroalgae) species that causes “green tides”. This alga inhabits the estuarine areas that exhibit changes in nutrient contents, which include changes in nitrogen (N) levels, while the mechanisms through which these microalgae resist N deficiency remains unclear. Results: We amplified the full-length sequences and quantified expression of genes involved in the nitrogen metabolism process, the data indicated that nitrate reductase, nitrite reductase, and glutamine synthase increased after nitrogen deprivation in Ulva prolifera. Hence, although the ratio of cell-wall regeneration did not change, the apoptosis rates of protoplasts of Ulva prolifera increased after this deficiency. Furthermore, a decreased in N supplies triggered the activation of MAPK signaling, and SB239063, a p38 MAPKα/β inhibitor, enhanced the effects of N deficiency on the mortality of protoplasts and decreased the capacity for cell-wall regeneration. Conclusions: All the data provided evidence that MAPK signaling had functional roles in helping U. prolifera adapt to fluctuations in N availability within a short time. Hence, the application of biochemical reagents on cell-wall regeneration on the surface of protoplasts provided a new perspective in the genetic breeding of Ulva prolifera.

Background

Green seaweeds or “macroalgae”, commonly termed “green tide”, first began to grow excessively along the shores of industrialized countries in the 1970s [1], and this condition has been observed since 2008 along the coastal zones of the western Yellow Sea in China [2, 3]. *Ulva prolifera* was identified as the main causative species of the largest recorded green tide in China [4], which was attributed to its strong capacity for nutrient uptake [5]. The nutrient uptake and growth rate of *Ulva* were reported to be faster than those in other macroalgae [6], the maximum rates of which were observed specifically in *U. prolifera* [7]. In addition to its reproductive strategies [8], reproducing both sexually and asexually to adapt to rapid proliferation under different conditions [9], *U. prolifera* also exhibits different metabolic activities that can help it acclimate to a fluctuating environment. For example, *Ulva* species displays greater nitrate reduction activity than other algae [10]; therefore, algal blooms, including macroalgal proliferation, have been suggested to be connected to the high nitrate concentrations [11], but the mechanisms by which nitrate assimilates in the species that form green tides has been unclear.

Among the major sources of nitrogen (N) for plant growth and development, nitrate plays a more important role than ammonium. In plants, nitrate reductase (NR) and nitrite reductase (NiR) catalyze nitrate into nitrite, glutamine synthetase (GS), and glutamine oxoglutarate aminotransferase (GOGAT) and are involved in ammonium assimilation [12]. The function of these enzymes contributes to eliminating the toxic effects of nitrite and ammonium produced under biotic and abiotic stress. For example, 50 min of N depletion enhances the levels of NR activity in *Ankistrodesmus braunii* [13]; NR mRNA accumulation fluctuates during a light–dark cycle in both tomato and tobacco plants, which rapidly increases during dark periods and reaches a maximum at the beginning of the light period [14]. NR establishes the connections between nitrate assimilation and photosynthesis in a number of unicellular marine algae under N-limiting conditions, which promotes the uptake of inorganic and organic

N [15]. Although these enzymes have been widely identified in higher plants, because of poor genome and molecular studies on algae, this is the first time that the functional effects of *UpNR*, *UpNiR*, and *UpGOGAT* have been demonstrated to prevent N deficiency from having toxic effects on *U. prolifera*.

Hence, except for the genes mentioned above, some nutrient signaling has also been intensively studied during recent years [16, 17]. For example, an increase in the steady-state level of mRNA was observed for MPK11 and MKK9 in response to N deficiency [18]; N deficiency was observed to induce Ras 1 phosphorylation at Ser-226 residues, which regulate the growth and proliferation of budding yeast *Saccharomyces cerevisiae* [19]; the dephosphorylation of Sch9, a direct substrate of the yeast multiprotein complex target of rapamycin (TORC1), was observed to prevent TORC1 signaling during N deficiency, the activation of which contributes to the synthesis of proteins and, in turn, cell growth [20]. Among these activated or inactivated nutrient-signaling pathways during N deficiency, MAPK was the most well-known in higher plants; however, the number of mitogen-activated protein kinases in algae have never been reported. Based on the transcription results reported in our previous study [21], the aim of our study was to investigate the role of the MAPK signaling pathway in mediating defenses against N deficiency in *U. prolifera*.

Materials And Methods

Plant materials and growth conditions

Algal materials of floating *U. prolifera* was collected from the coast of Qingdao (36°48'39.75"N; 121°38'10.88"E) on 10th, May, 2018, which was approved by Qingdao Municipal Marine Development Bureau (Qingdao, China). Then the alga was pre-cultured at 20℃ in SPX-GB-250 intelligent illumination incubators (Botai, Shanghai, China) for 4 d in fresh distilled seawater supplemented with macro-elemental solution as previously reported [21], and the voucher specimens was maintained in fresh distilled seawater at 4℃. *Ulva prolifera* used for the further experiment was confirmed by both morphology and molecular analysis by Yi Yin according to the previous study[22, 23]. The experiment was conducted according to the criterion of Convention on the Trade in Endangered Species of Wild Fauna and Flora.

After pre-cultural treatment, the *U. prolifera* was cultured in different medium created according to the study [24]. The composition for the pre-cultivation (N sufficient) or N-deficient medium consisted of the following: 450 mM NaCl 450 (pre-cultivation); 16.8 mM KNO_3 (pre-cultivation), 0 KNO_3 (N deficient); 0 KCl (pre-cultivation), 33.6 mM KCl (N deficient); 3.5 mM Na_2SO_4 (pre-cultivation and N deficient); 100 mM 2-[4- (2-hydroxyethyl) piperazin-1-yl ethanesulfonic acid (HEPES); 5 mM $MgSO_4{\cdot}7H_2O$ (pre-cultivation and N deficient); 2.5 mM $CaCl_2{\cdot}2H_2O$ (pre-cultivation and N deficient); 2.5 mM K_2HPO_4; 10 mM $NaHCO_3$; 28 μM NaFe ethylenediaminetetraacetate (NaFeEDTA); 80 μM $Na_2EDTA{\cdot}2H_2O$; 19 μM $MnCl_2{\cdot}4H_2O$; 4 μM $ZnSO_4{\cdot}7H_2O$; 1.2 μM $CoCl_2{\cdot}6H_2O$; 1.3 μM $CuSO_4{\cdot}5H_2O$; 0.1 μM $Na_2MoO_4{\cdot}2H_2O$; 0.1 μM Biotin, 3.7 μM vitamin B1; and 0.1 μM vitamin B12. The pH was adjusted with NaOH to 7.5. The high buffer capacity of

the medium ensured that any change in pH value was never >0.6 units in the N-sufficient cultures, and that the pH did not change in N-deficient cultures.

Determination of growth rates and chlorophyll contents

Approximately 0.2 g *U. prolifera* was placed into 1-L glass flask in incubators and cultured in N-sufficient or N-deficient medium; each treatment was conducted in triplicate. At the indicated time point, the algal material was reweighed and the relative growth rates (RGR) were calculated as follows:

$$RGR = (\ln w2 - \ln w1) / \Delta t,$$

where w1 is the initial fresh weight and w2 the fresh weight after Δt days.

For chlorophyll detection, an approximately 0.1 g sample was ground in liquid nitrogen, weighed, and suspended in extraction buffer (80% v/v acetone). After incubation on ice for 15 min, the samples were centrifuged at 6000 rpm at 4℃ for 10 min. Chlorophyll concentrations (Chla and Chlb) were determined according to the methods described by Arnon [25]. The equations used were as follows:

$$Chla = (12.71 \times A663 - 2.59 \times A645) \times V / (W \times 1000), \text{ and}$$

$$Chlb = (22.88 \times A645 - 4.67 \times A663) \times V / (W \times 1000),$$

where V represents the volume of extraction buffer, and W represents the weight of the algal sample.

RNA extraction and quantitative reverse-transcription polymerase chain reaction analysis

Ulva prolifera was harvested at the indicated time point. After grinding in liquid nitrogen, RNA was extracted using TRIzol reagents (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was purified using RNA resuspension buffer to remove any polysaccharides, as per the manufacture's protocol [26]. Two micrograms of RNA were reversed using the Hifair™ II 1st Strand cDNA Synthesis Kit (Yeasen, Shanghai, China). The program was set as 25℃ for 5 min, 42℃ for 60 min, and 85℃ for 5 min. Gene expressions were detected using the Hieff® qPCR SYBR Green Master Mix (Low Rox Plus) (Yeasen) and calculated as described by Liao *et al.* [27]. The specific primer pairs used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) are listed in Table 1; 18S rRNA was used as the internal controls. The $2^{-\Delta\Delta CT}$ method [28] was used to analyze the expression levels of the indicated genes.

Table 1 Primers for qRT-PCR and PCR analysis

Primers name		Sequences (5' to 3')
*Up*NR	Forward	CTGGATCGGTGGTCGTATGG
	Reverse	AGCACCTTGTTGTCGTGGAA
*Up*NiR	Forward	CAACGTTCGCAACCTGACTG
	Reverse	GCAGATGTTGATCTTGCGGG
*Up*GOGAT	Forward	TCTGCGACCTGCTATTGCTC
	Reverse	AGCCGCTAGTCCACAAGAAC
18S rRNA	Forward	CGCAACTCCCGACTCACGAAGG
	Reverse	ACCGGACCATGTGGCCCAGAAT
*Up*NiR$_{\text{-PCR}}$	Forward	GGGGCTGATTGAAACGCAGTAG
	Reverse	AACGGAGCCATCACATCACCC
*Up*GOGAT$_{\text{-PCR}}$	Forward	CTAGGCGGACCTTTGCAGTGTTCTAT
	Reverse	TCGAGCTTCTTCATCGGCGTGT

Full-length complementary DNA cloning and bioinformatics analysis

Because the sequence of *UpNR* from *U. prolifera* had been successfully cloned [29], we further designed primers for *UpNiR* and *UpGOGAT* based on the UniGene sequence from the transcriptome results of our previously study [21]; the primer pairs are listed in Table 1. The cDNA template was constructed as in previous studies, and partial sequences for these genes were obtained using 2 × Hieff Canace® PCR Master Mix (Yeasen). *UpNiR* and *UpGOGAT* PCR amplifications were conducted at 94°C for 5 min, with 35 cycles (94°C for 45 sec, 58°C for 60 sec, and 72°C for 90 sec). PCR amplifications for *UpGOGAT* were conducted at 94°C for 5 min, with 35 cycles (94°C for 45 sec, 58°C for 60 sec, and 72°C for 3 min), followed by an extension reaction at 72°C for 5 min. PCR products were gel-purified in 1% agarose gel, and the target fragment excised and cloned into a pESI-T vector (Yeasen). After transforming the fragments into competent *Escherichia coli* cells, positive recombinants were identified using PCR, and the clones were sequenced for verification (Invitrogen).

The *UpNiR* and *UpGOGAT* gene sequences were analyzed using the BLAST algorithm at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast). A phylogenetic tree was constructed using the neighbor-joining method with Mega 5.0 (http://www.megasoftware.net).

Protoplast isolation, Fluorescent Brightener 28 and Evans Blue staining

Approximately 1.0 g *U. prolifera* was cut and chopped into 0.5 - to 1.0-mm strips and immediately transferred into a deep petri dish containing WS1 buffer solution (0.5 M mannitol, 4 mM MES-KOH [pH 5.5], and 20 mM KCl). After shaking for 3 min, the liquid was removed, and 5 mL ES buffer (1.5% w/v cellulase R10, 0.4% w/v Macerozyme R10, 0.4 mM mannitol, 20 mM KCl, 20 mM 2-(N-morpholino) ethane sulfonic acid, pH 5.5 heated to 55℃ for 10 min and cooled to room temperature before adding 10 mM $CaCl_2$ and 0.1% BSA). The algae were digested in ES buffer while shaking at 60-80 rpm for 5 h at 28℃. After enzymatic digestion, an equal volume of WS2 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES at pH 5.7) was added and the protoplast was filtered through 120-μm and 40-μm nylon mesh sieves. After configuring at 50 × g for 10 min, the protoplasts were washed twice with WS2 buffer, resuspended into the N-sufficient or N-deficient medium, and cultured separately in the dark for 24, 48, 72, and 96 h.

To determine the viability of the cells, we used Evans Blue staining to estimate the percentage of viable protoplasts before or after exposure to N-deficient medium. For this, 1 μL 1% Evans blue was added to an aliquot of 25 μL protoplasts and incubated for 5 min at room temperature, after which the protoplasts were detected using a light microscope. The percentage of dead protoplasts that were stained with Evans Blue was calculated using the ratio of stained to total protoplasts.

To determine the percentage of protoplasts capable of regeneration, a known number of protoplasts were spread evenly over (or suspended in) a liquid medium in 24-well plates. Samples of regenerating protoplasts were centrifuged at 3,000 × g for 5 min and the resulting pellet suspended in a 0.1% (w/v) solution of Fluorescent Brightener 28 (Sigma-Aldrich, St. Louis, MO, USA). After incubating for 5 min at room temperature, the samples were examined under oil using the Lecia fluorescence microscope according to the manufacture protocol [30].

Protein extraction and Western blot analysis

At the indicated time point, *U. prolifera* was harvested and ground in liquid nitrogen. The algal powder was put into a radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) with Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific) at 4℃ for 30 min. After centrifuging at 120,000 × g at 4℃ for 15 min, the suspension was collected and desaturated at 100℃ for 5 min.

Approximately 30 μg protein was separated with 12% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking in 5% nonfat milk diluted in TRIS-buffered saline and Tween 20 (TBST) for 1 h at room temperature, the membranes were incubated at 4℃ overnight with the following primary antibodies: phospho-p38 MAPK (Thr180/Tyr182) (3D7) rabbit mAb (dilution, 1/500; Cell Signaling Technology, Danvers, MA, USA), p38MAPK antibody (dilution, 1/500; Proteintech, Wuhan, China), β-actin antibody (dilution, 1/2500; PhytoAB, San Francisco, CA, USA). The membranes were then washed three times with TBST and the secondary antibodies (horseradish peroxidase conjugated goat anti-mouse IgG (H + L), or goat anti-mouse (H + L) purchased from Proteintech, Co., Ltd.) were laid separately on the membranes for

1 h at room temperature. Protein was detected using enhanced chemiluminescence reagents (Thermo Fisher Scientific), and analyzed using Image J (National Institute of Health, Bethesda, MD, USA); β-actin was used as the internal controls.

Statistical analyses

Results of all experiments are expressed as the means ± standard deviation (SD). $P \leq 0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad Prism (https://www.graphpad.com/scientific-software/prism/).

Results

N deficiency decreased the chlorophyll content and changes in wet weight

The growth ratio of *U. prolifera* in N-sufficient or N-deficient medium was observed for 6 d and the algae weights were recorded every 2 d. RGR of *U. prolifera* in N-deficient medium declined after the second day compared with that in the pre-cultivation medium (Figs. 1-A); however, N deficiency had no effect on the levels of Chla or Chlb, but the values of total chlorophyll decreased on the second day, which resulted from the decline trend of Chla and Chlb (Figs. 1-B, C, D).

*Up*NiR, and *Up*GOGAT sequence analyses

To verify the accuracy of sequences of *Up*NiR and *Up*GOGAT from the transcriptome results, we synthesized partial CDS with 1833 bp of 2496 bp *UpNiR* and 2683 bp of 4833 bp *Up*GOGAT fragments (Figs. 2-A). Nucleotide sequence analysis indicated that 1833 bp of *Up*NiR and 2683 bp of *Up*GOGAT were both in the open reading frame (ORF) and encoding amino acids. Hence, the *Up*NiR *and Up*GOGAT cDNA and amino acid sequences were deposited in NCBI GenBank with accession number MN496140 and MN496141, respectively.

Additionally, the amino acid sequences of indicated molecular were used to constructed a phylogenetic tree, the results showed that the phylogenetic positions of *Up*NiR was clustered into one subgroup with NiR from *Chlamydomonas reinhardtii*, and into adjacent subgroup with NiR from *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Solanum tuberosum*, while *Up*GOGAT was clustered into one subgroup with the GOGAT from *Coccomyxa subellipsoidea* and adjacent subgroup with GOGAT from *C. reinhardtii*, and *Volvox carteri f. nagariensis* (Figs. 2-B, C).

*Up*NR, *Up*NiR, and *Up*GOGAT expression is induced by N deficiency

In higher plants and algae, NR synthesis is highly regulated, especially by sources of N, and our data demonstrated that the expression of *Up*NR, *Up*GOGAT, and *Up*NiR was stably maintained after exposure to N deficiency for 2 d; however, after 4 d., N deficiency induced the expression of *Up*NR and *Up*NiR, which suggested that both *Up*NR and *Up*NiR were the previous enzymes that responded to the N deficiency (Figs. 2-D, E). Hence, prolonged N deficiency led to significantly higher levels of *Up*GOGAT on day 6 (Figs.

2-F). All the data suggested that *U. prolifera* might resort to internal N sources, such as proteins and amino acids, to respond to abiotic stress; however, the mechanism by which this process is conducted in *U. prolifera* remains unclear.

N-deficiency induces protoplast apoptosis but does not reduce cell-wall regeneration

Most of the freshly isolated protoplasts were 7–15 and 30–40 μm in diameter and rich in pigment. These protoplasts that were isolated using Fluorescent Brightener 28 staining showed no trace of blue fluorescence when examined under an ultraviolet florescence microscope, and dead protoplasts were stained dark blue with Evans Blue (Figs. 3).

To investigate the regeneration of protoplasts in different culture media, approximately 5×10^4 cells/well were plated onto 24-well plates and stained with Evans Blue and Fluorescent Brightener 28 for 1, 2, 3, or 4 d. The results showed that after incubating for 2 d, new cell walls began to synthesize and that the protoplasts cultured in N-deficient medium showed high mortality on day 4 followed by those exposed to N-sufficient medium (Figs. S1, Figs. 4-A). However, no significant changes in cell-wall regeneration ratios were observed between N-sufficient and N-deficient medium, even in those protoplasts exposed to N-deficient stress for 4 d (Figs. S2, Figs. 4-B). Thus, we hypothesized that *U. prolifera* might evolve some unique mechanisms by which to resist the stress caused by this nutrient deficiency.

N deficiency activated the MAPK signaling pathway

Among the reported activated or inactivated signaling pathways under N-deficient conditions, MAPK was the most well-known in higher plants. In this study, we verified the role of MAPK signaling in *U. prolifera* cultured in either N-sufficient or N-deficient medium. The data indicated that N deficiency induced phosphorylated MAPK according to exposure time, and that treatment with 10 μM SB239063 (a p38 MAPKα/β inhibitor) led to a decline in MAPK levels (Figs. 4-C, D). In addition, pretreatment with 10 μM SB239063 significantly enhanced the mortality of the protoplasts after exposure to N deficiency. Hence, SB239063 relieved the detrimental effects on cell-wall regeneration on the surface of protoplasts compared with those kept in N-deficient medium (Figs. 5, Figs. 6 and Figs. 7). These data suggested that MAPK signaling plays functional roles in helping *U. prolifera* adapt to the fluctuations in N availability.

Discussion

To our knowledge, N is the most important nutrient for plants and is an essential component of key macromolecules, including proteins and nucleic acids [31]. In addition, it is widely accepted that the N content in the Yellow Sea is essential for supporting the world's largest green tides [11, 32]; therefore, it is critical that we identify the mechanism by which N triggers *U. prolifera* blooms. For plants, nitrate and ammonium are the vital sources of N, although the metabolic pathways are different from those in algae. In the cytosol and chloroplasts, nitrate reduction to nitrite followed by nitrite reduction to ammonium is catalyzed by NR and NiR, respectively [33]. Ammonium is assimilated to produce different amino acids by the combined action of GS and GOGAT in a cycle manner [34].

Balotf *et al.* (2016) have demonstrated that NR in plants is capable of catalyzing nitrite into nitric oxide (NO), which could be strengthened, particularly in the presence of high nitrite but low nitrate concentrations. Pre-growth in N-deficient medium resulted in a low beginning value of NR transcription, which may increase following fluctuations [35]; therefore, many studies have examined the NR clone from higher plants and algae, but the investigation of the *Up*NiR and *Up*GOGAT from *U. prolifera* has been minimal. In the present study, the full-length cDNAs of *Up*NiR and *Up*GOGAT were successfully cloned, and BLAST analysis confirmed that *Up*NiR and *Up*GOGAT were similar to the NiR from *C. reinhardtii*, and GOGAT from *Auxenochlorella protothecoides*, respectively. Hence, N deficiency induced the expression of *Up*NR and *Up*NiR in *U. prolifera* after exposure for 4 d and then reduced the expression to levels cultured in N-sufficient medium; however, the levels of *Up*GOGAT peaked at day 6. Based on these findings, we put forward the hypothesis that, in the initial 4 d, the increased *Up*NiR and *Up*NR might use proteins or amino acids as the N sources, then the metabolically produced ammonium is assimilated by *Up*GOGAT, which is why *Up*GOGAT peaked at day 6, after the occurrence of *Up*NR and *Up*NiR.

Hence, protoplasts were considered to generate when they could increase into a visible colony on an agar plate and then spread over the agar's surface. Successful regeneration required the use of a suitable inorganic salt, such as NaCl, $MgSO_4$, and KCl, and N sources; therefore, the adaption of protoplasts in response to N deficiency could partly explain why *U. prolifera* maintained steady growth under fluctuating N sources. In this study, we successfully created protoplasts of different diameters, which began to form the cell wall after culturing for 2 d. Although N deficiency enhanced the mortality of the protoplasts compared with that in N-sufficient medium, but once the surviving protoplasts began to form a cell wall, the N nutrient had no effect on them. This might explain why N deficiency had no influence on cell-wall regeneration on the surface of protoplasts, even after 6 d.

It is widely accepted that N content in the Yellow Sea is essential to the support of the world's largest green tides [11, 32]. Although the augments of *Up*NR, *Up*NiR, and *Up*GOGAT induced by N deficiency, as mentioned above, illustrated the potential possibility of the consumption of internal *U. prolifera* proteins as a defense against changing N contents within the environment, identifying the possible molecular mechanisms involved is also of great interest. It is well established that MAPK signaling mediates the process of cell growth and division in response to changes in environmental conditions, and exposure to the nutrient deficient environment leads to the activation of MAPK signaling in some plants [18]. Similarly, in our study, we found that N deficiency enhanced the levels of phosphorylated MAPK, and that this could be reversed by adding 10 μM SB239063. In addition, continual treatment with the MAPK signaling inhibitor led to the increased mortality of protoplasts and delayed the progression of cell-wall regeneration. In *Arabidopsis*, nitrate reductase 2 could be phosphorylated by both MPK7 and MPK6 [36, 37], meanwhile, both increased levels of NR and the activation of MAPK were identified in this study. Whether NR is the key that switches genes on and off for MAPK signaling in *U. prolifera* also needs further study.

Conclusions

This is the first report on a macroalgae that advances our understanding of the N-assimilation mechanism in *Ulva* and successfully cloned the N assimilation–associated enzymes. The activation of MAPK signaling might help *U. prolifera* adapt to fluctuations in N availability; therefore, the application of biochemical reagents on cell-wall regeneration on the surface of protoplasts provides a new perspective on genetic breeding of *U. prolifera.*

Abbreviations

U. prolifera, Ulva prolifera; MAPK, mitogen-activated protein kinase; NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; GOGAT, glutamine oxoglutarate aminotransferase; RGR, relative growth rates; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; HEPES, 2-[4- (2-hydroxyethyl) piperazin-1-yl ethanesulfonic acid;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the limitation of the upload space of website but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JJ. Y, Y. Y, and DC. Y performed the experiments, interpreted the results. JJ. Y and LH. H participated in the data analyses. JJ. Y wrote the manuscript. S. L and SD. S designed this study and revised the manuscript.

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Supplemental File Legends

Figure S1 Viable protoplasts stained by Evans blue in pre-cultivation and N-deficient medium.

Figure S2 Protoplasts stained by Fluorescent Brightener 28 under UV florescence microscope in pre-cultivation and N-deficient medium.

Figures

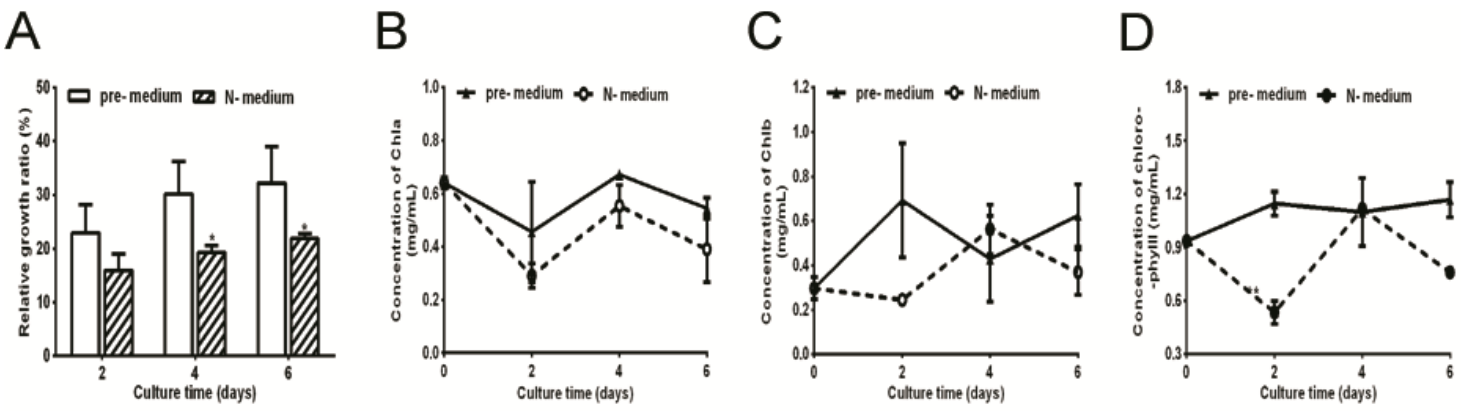


Figure 1

N deficiency decreased the chlorophyll content and changes in wet weight. (A) Growth ratio of U. prolifera in N-deficient medium and pre-cultivation medium. (B-D) Contents of Chla, Chlb and total chlorophyll in U. prolifera. **P0.01, vs pre-cultivation medium group.

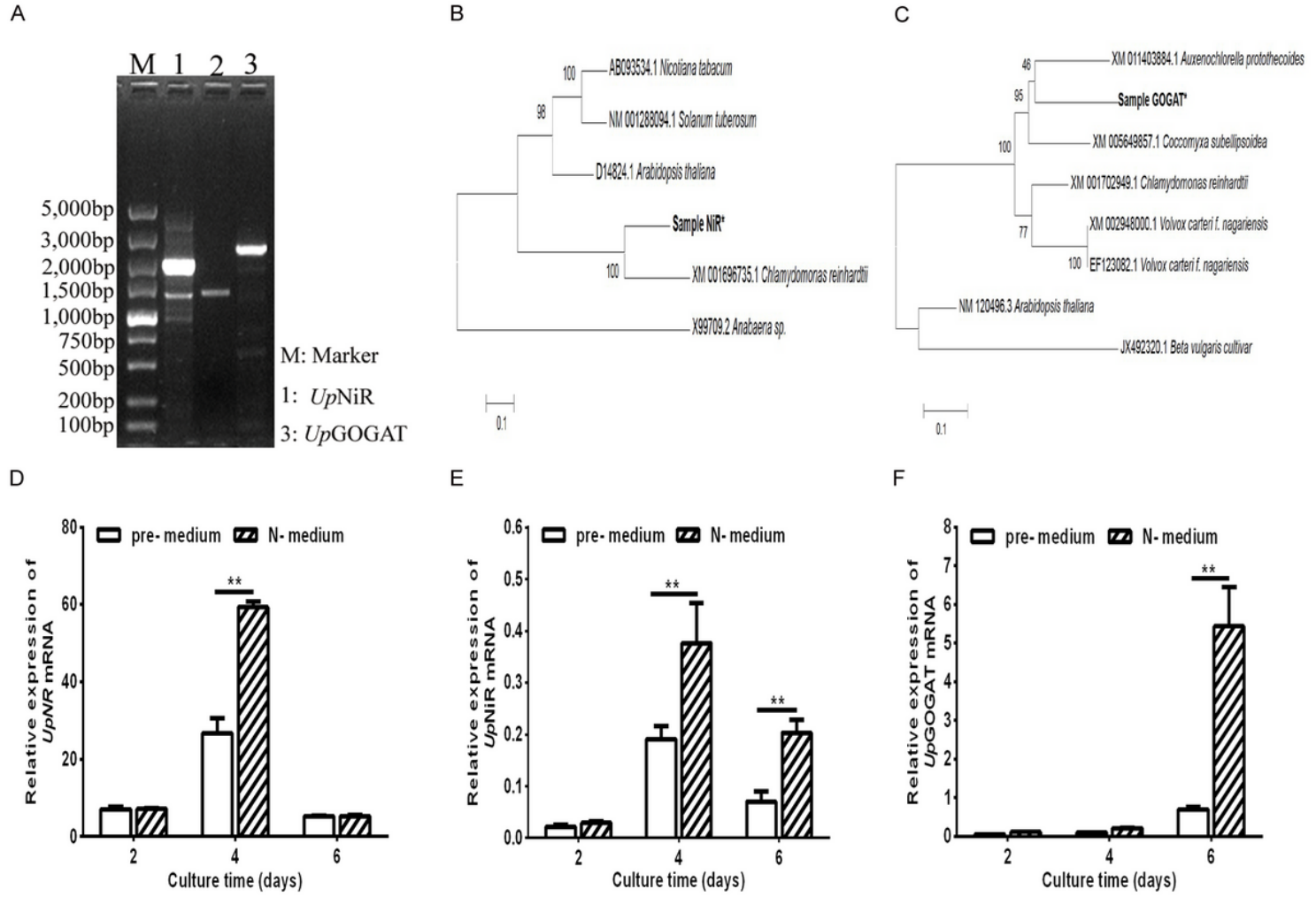


Figure 2

UpNiR, and UpGOGAT sequence analyses and expression. (A) Polymerase chain reaction indicated the partial CDS fragments of UpNiR and UpGOGAT. (B-C) The topology tree of UpNiR and UpGOGAT. (D-F) Expression of UpNR, UpNiR and UpGOGAT in U. prolifera exposed in pre-cultivation and N-deficient medium. *P0.05, **P0.01, vs pre-cultivation medium group.

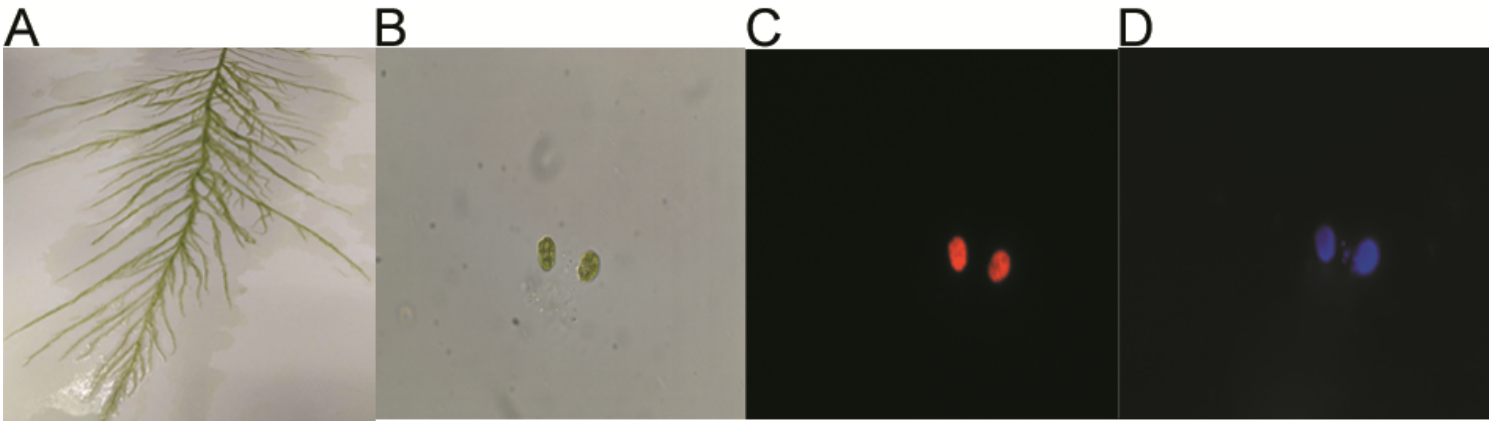


Figure 3

Protoplasts of U. prolifera. (A) Freshly U. prolifera thaills. (B) Freshly isolated protoplasts under light microscope. (C-D) Freshly isolated protoplasts stained with Fluorescent Brightener 28 under UV florescence microscope.

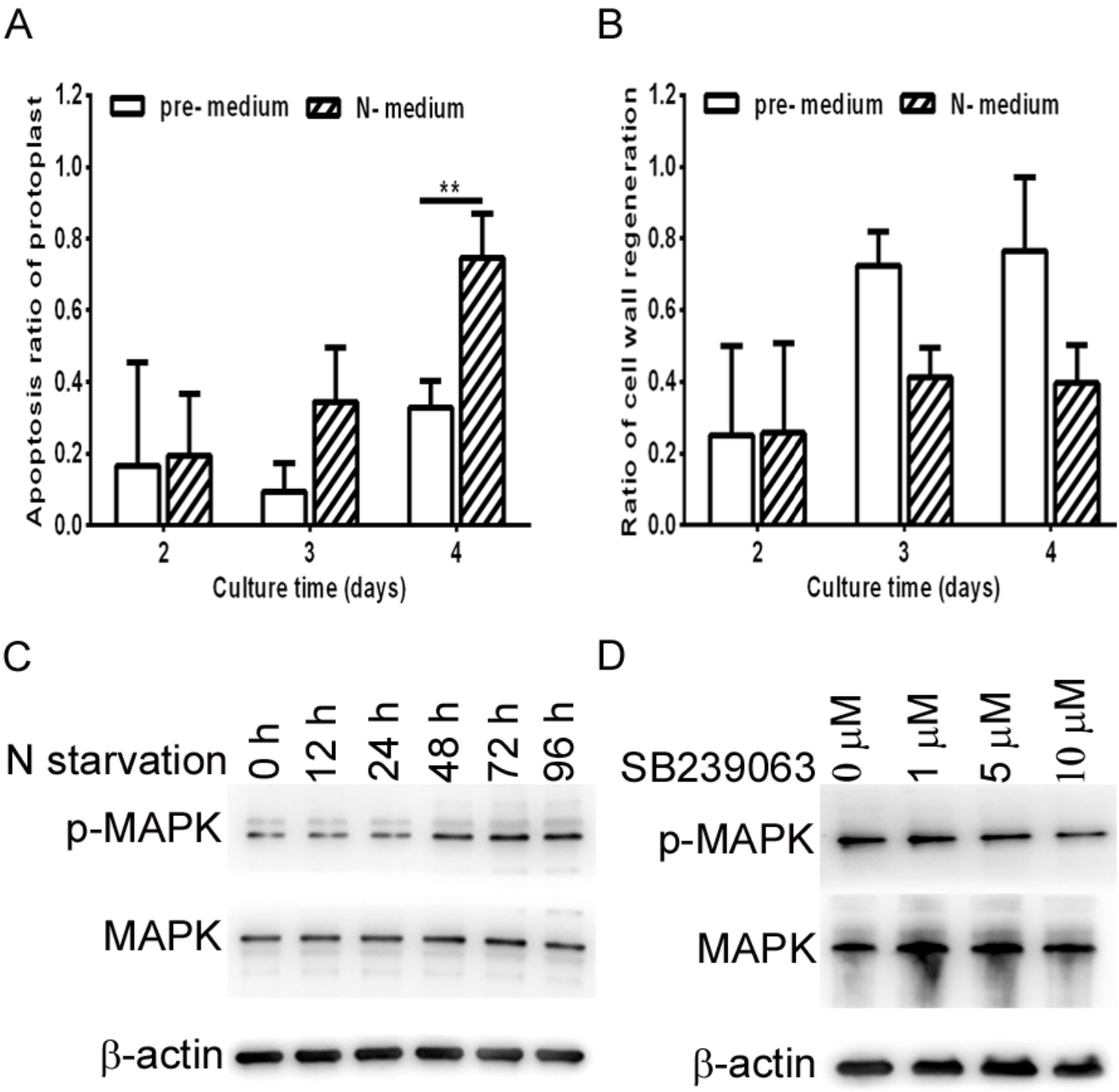


Figure 4

Nitrogen deficiency activated the MAPK signaling pathway. (A-B) Statistics analyses for the viability and cell wall regeneration of protoplasts in pre-cultivation and N-deficient medium. (C) N deficiency induced phosphorylated MAPK according to exposure time. (D) SB239063 (a p38 MAPKα/β inhibitor) led to a decline in phosphorylated MAPK levels.

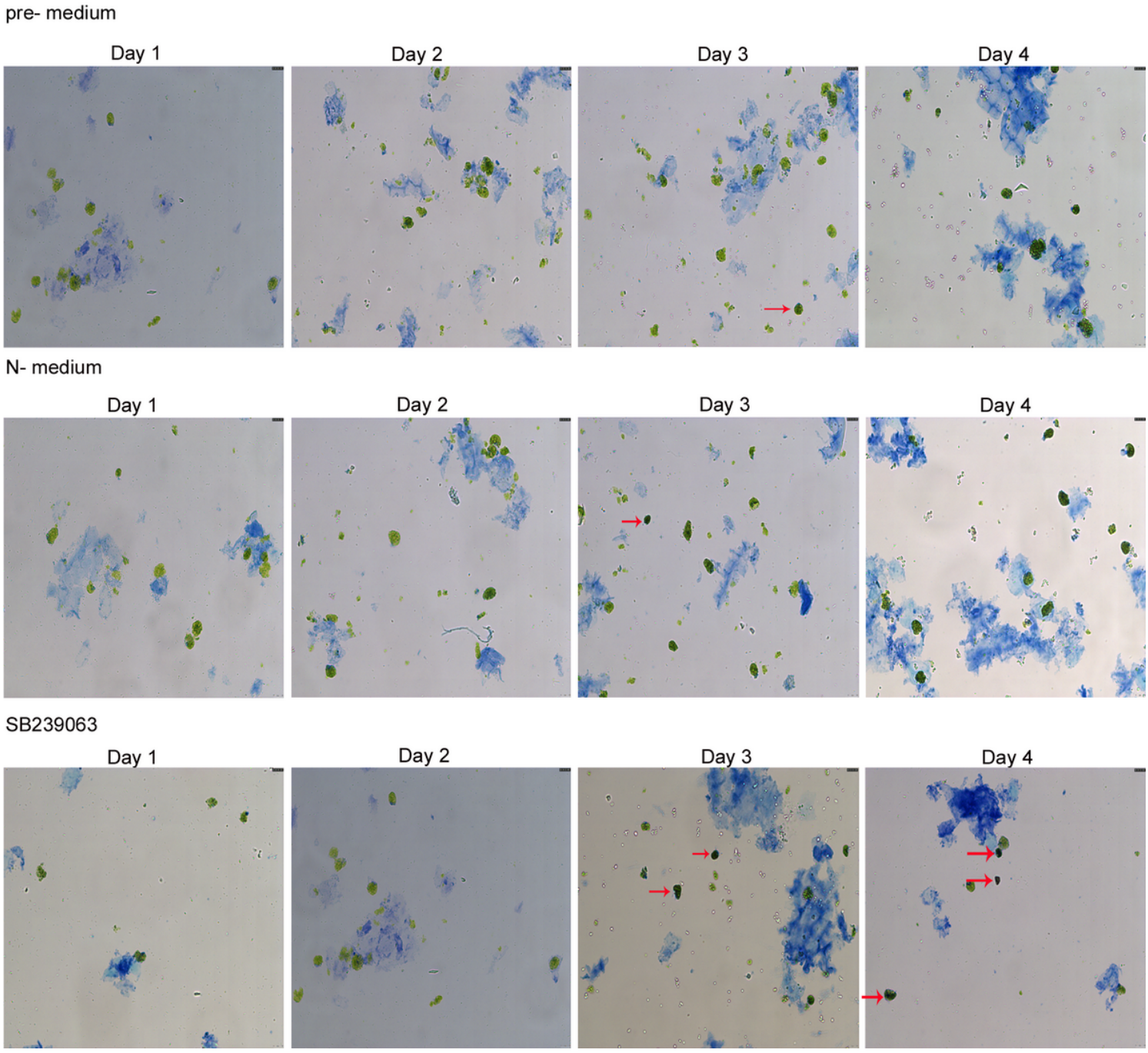


Figure 5

SB239063 enhanced the mortality of the protoplasts after exposure to N deficiency.

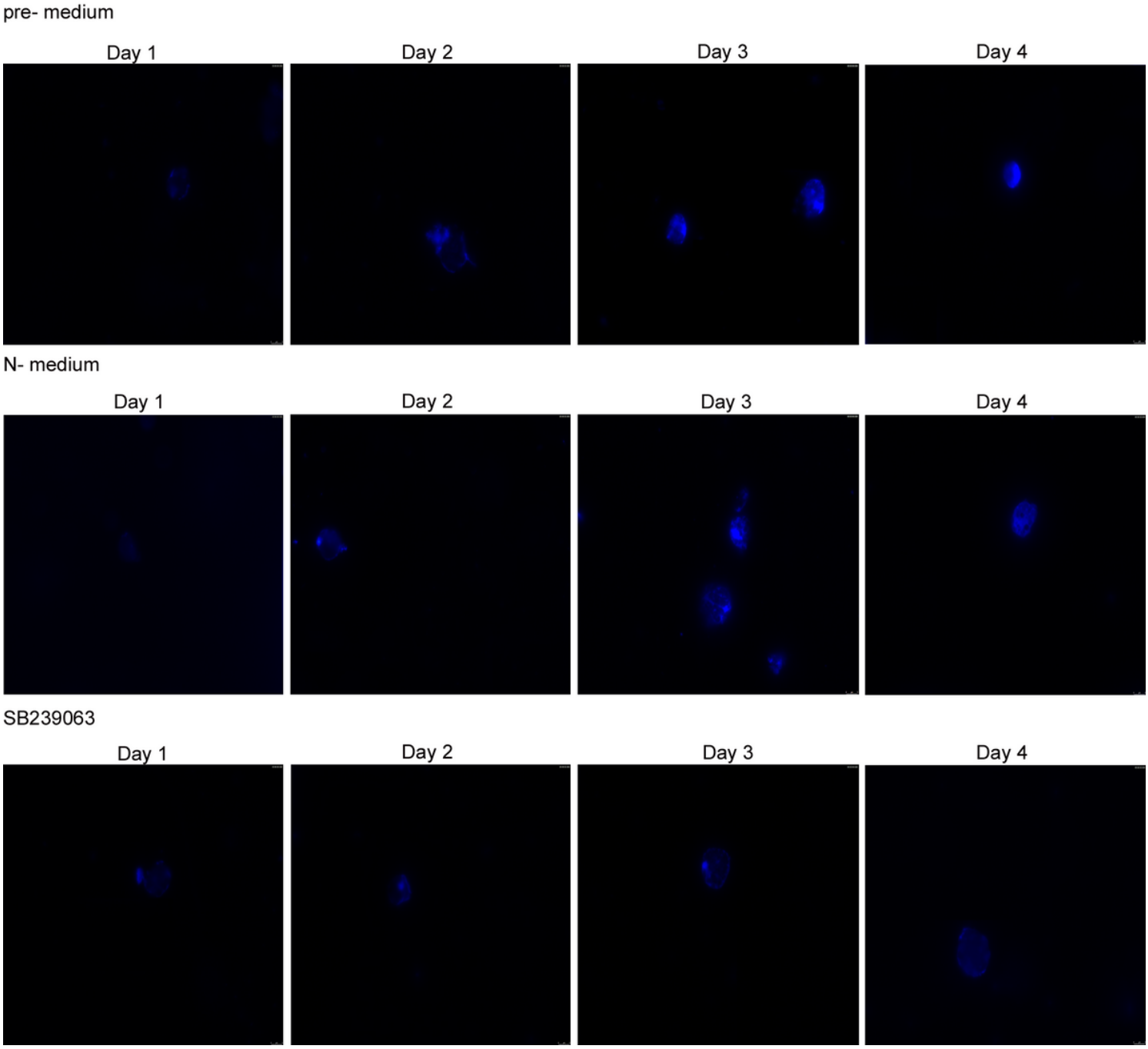


Figure 6

SB239063 relieved the detrimental effects on cell-wall regeneration on the surface of protoplasts compared with those kept in N-deficient medium.

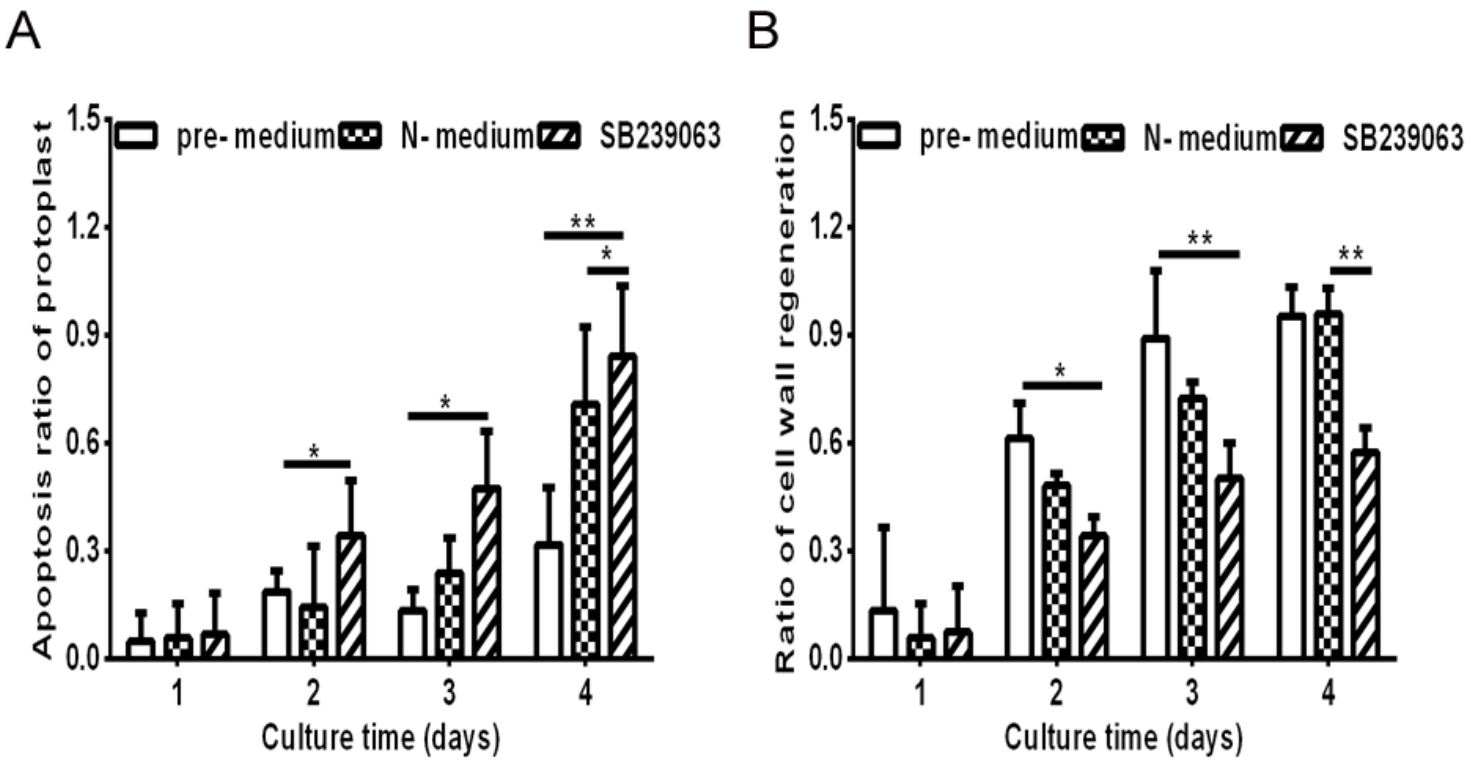


Figure 7

SB239063 enhanced the mortality of the protoplasts (A) while inhibited cell-wall regeneration (B) on the surface of protoplasts.

Supplementary Files

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- FigureS1.tif
- FigureS2.tif.tif