First evidence of domoic acid production in *Pseudo-nitzschia calliantha* cultures from the central Adriatic Sea

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In this study, three isolates of the potentially toxic diatom genus *Pseudo-nitzschia* were analysed for morphological and toxicological features. Cultures of *Pseudo-nitzschia* were established from seawater samples collected from the southern part of the Velebit Channel (central Adriatic Sea) during February 2019. All culture isolates were identified by scanning electron microscopy (SEM) as *Pseudo-nitzschia calliantha*. Domoic acid (DA) production was confirmed in all isolates analysed. The highest concentrations of cellular DA were found in early culture stages, with the lowest cell abundance, for all *P. calliantha* isolates. This study is the first to report DA production by *P. calliantha* isolated from the Adriatic Sea.

Key words: *Pseudo-nitzschia*, morphology, toxicity, domoic acid

INTRODUCTION

Diatoms of the genus *Pseudo-nitzschia* are common constituents of the marine phytoplankton community and regularly occur throughout the world’s oceans (LELONG et al., 2012; BATES et al., 2018), including the Adriatic Sea (VILIČIĆ et al., 2007; BUŽANČIĆ et al., 2012; MARIĆ et al., 2012; SKEJIĆ et al., 2014; NINČEVIĆ GLADAN et al., 2020). Due to its ability to produce the neurotoxin domoic acid (DA), *Pseudo-nitzschia* has gained considerable scientific attention. With the development of new molecular tools, research of *Pseudo-nitzschia* resulted in the discovery of many new species. LELONG et al. (2012) reported 37 *Pseudo-nitzschia* species, of which, 14 were toxic. To date, this genus comprises 60 described species, among which, 26 are confirmed toxic (BATES et al., 2018; HUANG et al., 2019; LUNDHOLM, 2019). Laboratory studies have shown that toxin production by *Pseudo-nitzschia* species is complex because species toxicity is influenced by abiotic (silicates or phosphates limitation, inorganic or organic nitrogen forms, temperature, salinity, irradiance) and biotic (bacteria, zooplankton) factors, as well as interactions between two or more factors (reviewed in LELONG et al., 2012; TRAINER et al., 2012; BATES et al., 2018).

Along the Croatian coast, the diversity of the *Pseudo-nitzschia* genus is based on the descriptions of 10 species (LJUBEŠIĆ et al., 2011; MARIĆ PFANNKUCHEN, 2013; ARAPOV et al., 2017; ARAPOV et al., 2019). The toxicity of these species has
not been reported, although low levels of DA were determined sporadically in shellfish from the eastern Adriatic coast (UJEVIĆ et al., 2010; LJUBEŠIĆ et al., 2011; ARAPOV et al., 2016; ARAPOV et al., 2017; UJEVIĆ et al., 2019). This study presents the first evidence of DA production in cultures of Pseudo-nitzschia species, in particular P. calliantha, in isolates from the eastern Adriatic coast.

MATERIAL AND METHODS

Cell isolation and SEM analysis

Pseudo-nitzschia calliantha strains were isolated from a phytoplankton net sample (20 µm pore size), collected at station M-S1 (44.2696°N, 15.51655°E) in February 2019 (Fig. 1). The net was towed between the surface and a depth of 7 m. Single-cell or chain was isolated under an inverted light microscope (Leica DMI4000B) using a sterile glass micropipette and transferred to 1 mL of f/2 medium in a 24-well tissue culturing plate. The plate was kept at 18 ± 0.5 °C, with a photoperiod of 12:12 h (light:dark) at 108 µmol photons m⁻² s⁻¹ for 7 days. Afterwards, the isolates were transferred to culturing flasks containing 30 mL of f/2 medium to increase density (initial flask). For morphological and toxicological analyses, three Pseudo-nitzschia calliantha cultures were successfully established: isolates D4, D5, and E2.

Morphological characteristics were examined using an SEM (Tescan, MIRA3). Subsamples of 10 mL each were taken from the initial flask and fixed with Lugol solution. Pseudo-nitzschia frustules were cleaned according to the method described in HASLE & FRYXELL (1970), filtered on polycarbonate membrane filters (pore size 1 µm, Nucleopore, Whatman), and dried in a desiccator for a minimum of 24 h. After that, filters were gold coated and analysed with the SEM.

Toxin analyses

For toxin analyses, 2 mL of each Pseudo-nitzschia isolate from the initial flask were inoculated into three flasks containing 80 mL of f/2 medium. To analyse the toxin content at different stages of culture, subsamples of 3 mL were collected on day 6 and day 13 for all isolates. Depending on cell density, additional subsamples were taken on days 22, 27, and 36 for isolates E2, D5, and D4, respectively. Subsamples of cultures were fixed with Lugol solution and cell abundance was analysed with an inverted light microscope (Leica DMI4000B) in a Sedgewick Rafter counting chamber.

At least 50 fields were counted per subsample, and empty frustules were not included in the count. On sampling days, the whole culture was filtered through GF/F filters (Whatman, pore size 0.7 µm) and frozen at -20 °C until toxin analysis. The exact volume of each filtered culture was measured, to calculate the total number of cells in the whole culture, and analysed for toxin content. At the time of analysis, filters were thawed and sonicated for 1 min in 5 mL of 100% MeOH, centrifuged at 2000 g for 10 min and filtered through 0.22 µm filters (FilterBio, Naylon Syringe Filter, 13 mm diameter).

The filtered methanol extracts of Pseudo-nitzschia species were analysed by liquid chromatography with tandem mass spectrometry (LC-MS/MS, Agilent Technologies) to determine DA content. The tandem mass spectrometer was equipped with a Triple Quad 6410, Degasser 1200, Quaternary Pump 1200, Autosampler 1290, and Thermostatted Column Compartment 1290. Chromatograph conditions for
the Poroshell 120 (EC-C18, 2.1 mm x 50 mm, 2.7 µm) column coupled to the Poroshell 120 (EC-C18, 2.1 mm x 5 mm, 2.7 µm) pre-column were: flow 0.3 mL/min, temperature 30 °C, and mobile phase B gradient from 10 to 80% in 4 min, held for 2 min, and recovered to initial condition for 5 min. Mobile phase A consisted of 100% water with 2 mM ammonium formate and 50 mM formic acid, while mobile phase B consisted of 95% acetonitrile, 5% water with 2 mM ammonium formate, and 50 mM formic acid. Quantification of DA by the multiple reaction monitoring mode (MRM) were performed in positive ion mode. Electrospray ionisation (ESI) was applied as the optimum ion source interface for DA. The identification of DA was based on the retention time of DA in HPLC and the exact of protonated parent ion (312.2 m/z) and the most intense product ion (266.1 m/z). For qualitative identification, a second selected fragment (248.0 m/z) based on intensity is required. Quantification of DA was performed using the calibration curves of six working standard solutions. The working standard solutions were prepared by diluting stock solutions containing a mixture of certified standards (DTXs, PTX-2, AZAs, SPX, GYM, and DA, National Research Council of Canada, Halifax, Canada). The concentration DA stock solution was 3000 ng mL⁻¹ and prepared in methanol. Six DA calibration working solutions were prepared in concentrations ranging between 30 and 450 ng mL⁻¹. The cellular DA concentration (ng DA cell⁻¹) was calculated by multiplying the DA concentration in the whole culture (ng mL⁻¹) with a volume of extraction solvent (5 mL) and dividing by the total cell abundance in the whole culture.

RESULTS AND DISCUSSION

Three clonal cultures were successfully established for morphological and toxicological analyses. Morphologically, cultures isolated from the Velebit Channel were confirmed as *Pseudo-nitzschia calliantha* (Fig. 2). For each isolate, 10 valves were measured, and morphological characteristics were observed by SEM and presented in Table 1. In the valve view, cells were linear and symmetrical (Fig. 2A). The transapical and apical axes of cultured cells were within the range 1.61–2.17 µm and 67.73–90.90 µm, respectively. A central interspace occupied 3.5 to 6 striae with central nodules (Fig. 2B). There were 17 to 21 fibulae and 32 to 37 interstriae per 10 µm, while the number of sectors within the poroids varied from 3 to 12. Sectors within poroids were arranged in a circle with 33–47% of poroids having a central sector and thus exhibiting the characteristic flower pattern (Fig. 2B). The structure of the girdle bands showed that the first band, the valvocopula, had 43 to 45 striae per 10µm. Each band stria was two to three poroids wide but varied in height. Valvocopula were 4 to 6 poroids high, while the second and third bands had arrangements of 3–4 and 2–3 poroids high, respectively (Fig. 2D). In comparison, cells from three established *P. calliantha* cultures showed similar morphological characteristics. Slight differences were observed for *P. calliantha* isolate D4, which had longer transapical axes, shorter apical axes, and fewer number of sectors within poroids than of isolates D5 and E2. The morphological characteristics of cultured *P. calliantha* (D4, D5, and E2) cells correspond to the original description by LUNDHOLM et al. (2003) and those previously observed from the Adriatic Sea (BURIĆ et al., 2008; LJUBEŠIĆ et al., 2011; MARIĆ et al., 2011; ARAPOV et al., 2016, 2017; TURK DERMASTIA et al., 2020) and the Mediterranean Sea (SAHRAOUI et al., 2009; MOSCHANDROU & NIKOLAIDIS, 2010; QUIJANO-SCHEGGIA et al., 2010). Identification of *P. calliantha* in this study was not confirmed by molecular tools; however, it shows good correspondence with morphological descriptions of previous findings (references herein) and it is commonly found in the eastern Adriatic.

Toxin analyses confirmed DA production by all three *P. calliantha* isolates analysed in this study. Cellular DA values ranged between 0.0022 and 0.0351 pg cell⁻¹ for the D4 isolate, 0.0032 and 0.0855 pg cell⁻¹ for D5, and 0.0038 and 0.0058 pg cell⁻¹ for E2 (Table 2). The highest concentrations of cellular DA were found in the early stages of development, with the lowest cell abundance, for all *P. calliantha* isolates.
Comparing the *P. calliantha* isolates analysed in this study, the highest DA content per cell was found in all stages of the D5 culture.

The species *P. calliantha* is a globally distributed *Pseudo-nitzschia* species, and although its toxicity has been reported from different areas, more studies cite non-toxic strains of *P. calliantha* (TRAINER et al., 2012; BATES et al., 2018 and references therein). Cellular DA content is reported within the range from 0.054 fg cell$^{-1}$ (WADT et al., 2017) to 0.95 pg cell$^{-1}$ (BESIKTEPE et al., 2008). Given the results of the toxin analyses, measured DA concentrations for *P. calliantha* isolates from the central Adriatic Sea were lower than those reported by LUNDHOLM et al. (1997) and cited in LUNDHOLM et al. (2003) at 0.221 pg cell$^{-1}$, BESIKTEPE et al. (2008) at 0.95 pg cell$^{-1}$, and STONIK et al. (2019) at 0.441 pg cell$^{-1}$, but consistent with values reported by ALVAREZ et al. (2009) at 0.01 pg cell$^{-1}$, THESSEN et al. (2009) at 0.001–0.0057 pg cell$^{-1}$, and STONIK et al. (2019) at 0.015–0.077 pg cell$^{-1}$.

Consistent with our findings, BESIKTEPE et al. (2008) recorded higher cellular DA levels during the early exponential phase of development, as reported for *P. cuspidata* (AURO & COCHLAN, 2013). In contrast, many studies confirmed higher DA production in the stationary phase, suggesting that the mechanism of DA production may differ among species and strains (BATES et al., 2018).

Although the presence of 15 potentially toxic *Pseudo-nitzschia* species were recorded in the Mediterranean Sea (ZINGONE et al., 2020), DA production was only confirmed for 7 species. The only two species from the Adriatic Sea confirmed to produce DA are *P. multistriata* (PISTOCCHI et al., 2012) and *P. delicatissima* (PENNA et al., 2013). In other parts of the Mediterranean, toxicity was recorded for *P. brasiliiana*, *P. calliantha*, and *P. cf. delicatissima* from Bizerte Lagoon (south-west Mediterranean, SAHRAOUI et al., 2009, 2011); *P. galaxiae*, *P. pseudodelicatissima*, and *P. pungens var. pungens* from Greek coastal waters (north-east Mediterranean, MOSCHANDREOU et al., 2010, 2012), and for *P. galaxiae* and *P. multistriata* from the Tyrrenian Sea (ORSINI et al., 2002; CERINO et al., 2005; AMATO et al., 2010).

To our knowledge, this study is the first report of DA production by *Pseudo-nitzschia calliantha* isolated from the Adriatic Sea. *Pseudo-nitzschia* species frequently occur in the Adriatic phytoplankton community with increasing abundance over the last 15 years (NIČEVIĆ GLADAN et al., 2020) and are occurring in all seasons (TURK DERMASTIA et al., 2020). Our results present new insight into the toxicity of this important diatom genus, which is still mostly unexplored along the eastern Adriatic coast.

**ACKNOWLEDGEMENTS**

We would like to thank Mr. Nikša NAZLIĆ for conducting field sampling. This study was fully funded by the Croatian Science Foundation.
Table 1. Morphological characteristics of three cultured strains of P. calliantha (isolates D4, D5, and E2). Number of measured cells was 10; minimum and maximum values are given in bold; average ± standard deviation values are specified row below. Number of measured poroids (n) is presented in parentheses.

<table>
<thead>
<tr>
<th>Species (n)</th>
<th>Width (μm)</th>
<th>Length (μm)</th>
<th>CN</th>
<th>Fibulae (10μm)</th>
<th>Interstriae (10μm)</th>
<th>Poroid rows</th>
<th>Poroids (1μm)</th>
<th>Sectors within poroid (n)</th>
<th>Band striae (10μm)</th>
<th>Structure of valvocopula (width x height)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. calliantha D4</td>
<td>1.72-2.17</td>
<td>67.73-83.63</td>
<td>4-6</td>
<td>17-20</td>
<td>32-37</td>
<td>1</td>
<td>4-5</td>
<td>3-10</td>
<td>43-45</td>
<td>2-3 x 4-5</td>
</tr>
<tr>
<td></td>
<td>1.92±0.13</td>
<td>80.23±4.69</td>
<td></td>
<td>18.70±1.06</td>
<td>34.70±1.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. calliantha D5</td>
<td>1.61-1.95</td>
<td>87.13-90.90</td>
<td>3.5-6</td>
<td>17-20</td>
<td>34-37</td>
<td>1</td>
<td>4-5</td>
<td>3-11</td>
<td>44-45</td>
<td>2-3 x 4-6</td>
</tr>
<tr>
<td></td>
<td>1.78±0.13</td>
<td>88.41±1.21</td>
<td></td>
<td>18.60±0.97</td>
<td>35.50±1.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44.33±0.51</td>
</tr>
<tr>
<td>P. calliantha E2</td>
<td>1.56-1.80</td>
<td>85.18-87.52</td>
<td>4-5</td>
<td>17-21</td>
<td>34-37</td>
<td>1</td>
<td>3.5-6</td>
<td>3-12</td>
<td>44-45</td>
<td>2-3 x 4-6</td>
</tr>
<tr>
<td></td>
<td>1.73±0.07</td>
<td>86.26±0.75</td>
<td></td>
<td>18.85±1.29</td>
<td>35.89±0.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44.30±0.48</td>
</tr>
</tbody>
</table>

Table 2. Concentration of domoic acid (DA) determined by LC-MS/MS in P. calliantha cultured strains (D4, D5, E2) at different cell abundances.

<table>
<thead>
<tr>
<th>Species</th>
<th>Culture age (days)</th>
<th>Cell density in culture (st mL⁻¹)</th>
<th>Cell abundance in whole culture</th>
<th>Average DA concentration in culture (ng mL⁻¹± SD)</th>
<th>DA concentration per cell (pg cell⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. calliantha D4</td>
<td>6</td>
<td>4840</td>
<td>266200</td>
<td>1.8671±0.9775</td>
<td>0.0351</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>52520</td>
<td>3098680</td>
<td>1.3431±0.3248</td>
<td>0.0022</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>138200</td>
<td>7255000</td>
<td>&lt; LOD</td>
<td></td>
</tr>
<tr>
<td>P. calliantha D5</td>
<td>6</td>
<td>2720</td>
<td>131920</td>
<td>2.2557±0.4433</td>
<td>0.0855</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>25800</td>
<td>1332820</td>
<td>2.2651±0.8495</td>
<td>0.0085</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>75060</td>
<td>3640410</td>
<td>2.3024±0.1343</td>
<td>0.0032</td>
</tr>
<tr>
<td>P. calliantha E2</td>
<td>6</td>
<td>6180</td>
<td>352260</td>
<td>0.5129±0.0762</td>
<td>0.0058</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>16180</td>
<td>857540</td>
<td>0.6472±0.1179</td>
<td>0.0038</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>16920</td>
<td>820620</td>
<td>&lt; LOD</td>
<td></td>
</tr>
</tbody>
</table>
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Prvi nalaz proizvodnje domoične kiseline u kulturama vrste *Pseudo-nitzschia calliantha* iz srednjeg Jadrana

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SAŽETAK


Ključne riječi: *Pseudo-nitzschia*, morfologija, toksičnost, domoična kiselina