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SHORT COMMUNICATION

Regional differences in aspen (*Populus tremuloides* Michx.) seedling response to an established nursery protocol

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Abstract

In seedling-based reforestation operations, seed source is known to be an influential variable affecting outplanting success. Adaptive variation among seed sources may also be an important factor in the effectiveness of standardized nursery protocols for seedling production. Particularly for wide-ranging species, regional optimizations of nursery protocols may be necessary to ensure consistent production of quality seedling stock. Quaking aspen (*Populus tremuloides* Michx.) is one such species, possessing the broadest distribution of any tree in North America. However, research on nursery protocols specific to aspen has focused on seed sources from a limited region in western boreal Canada. A well-established protocol shown to be effective for these boreal aspen uses a shoot growth inhibitor designed to maximize desirable seedling quality traits for outplanting success. We used this protocol on seeds sourced from two different regions in the southwestern portion of the species range (Utah and New Mexico, USA) and compared their response in the same nursery environment to that of a seedlot from Alberta, Canada to determine whether this protocol is also applicable for these very different regions. Seedlings from Utah and New Mexico differed significantly in their response to the protocol from the Alberta source, developing smaller root-to-stem ratios and sequestering less carbohydrate and nutrient reserves. Seedlings from Utah and New Mexico sources also differed from each other, with New Mexico seedlings growing larger according to all metrics. These results indicate that aspen nursery protocols will benefit from regional modification in order to optimize seedling stock quality and trait consistency.

Keywords Aspen restoration · US Intermountain West · Growth and carbon allocation · Nutrient reserves

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Introduction

Tree seedling production has been an important component of silvicultural practice for centuries (Evelyn 1664). In the past century, research has increasingly focused on growing trees for reforestation, with the goal of producing quality seedlings that maintain high survival rates along with production goals (Dumroese et al. 2016). For example, concerted efforts to develop nursery best practices for the economically important southern US pine species began in the 1920s and continued late into the century, resulting in survival rates for planted seedlings that now commonly exceed 90% (Fox et al. 2007). In recent decades, discussions about seedling quality have emphasized assessment of seedling performance at the outplanting site based on genetics (i.e., seed source) and specific seedling characteristics developed in the nursery (i.e., the "Target Plant Concept") (Landis 2011; Dumroese et al. 2016). One important element of the Target Plant Concept is that adaptive traits in the seed source are an aspect of seedling quality and should be matched with appropriate outplanting locations. However, less is known about the role that seed source plays on seedling development in the nursery. If seed source influences seedling responses to nursery practices, then regional adaptation of nursery protocols may be necessary, particularly for species with broad ranges or ecological amplitudes.

Quaking or trembling aspen (*Populus tremuloides* Michx.) is one such species, possessing the broadest range of any tree in North America (Little 1971). A pronounced phylogeographic boundary exists between southwestern and northern aspen populations, and populations within the southwestern region tend to show more differentiation than those in the boreal region (Callahan et al. 2013; Latutrie et al. 2016). This suggests aspen may possess regional differences among seed sources, particularly in the southwestern portion of its range. These differences may not only be a concern for seed transfer zones and assisted migration practices (Gray et al. 2011), but could also manifest themselves as different responses to nursery protocols.

To date, aspen seedling production has largely been limited to western Canada, where reclamation of industrial disturbed boreal sites has fueled extensive research on the use and quality of planted aspen seedlings in recent years (Macdonald et al. 2012). Unlike most conifer seedlings, for which aboveground traits such as root collar diameter (RCD) and height can be good indicators of seedling stock quality (Sutton 1979), root system traits appear to be more important indicators for aspen. In particular, high root-to-stem ratios (RSR), along with total non-structural carbohydrate (NSC) reserves and nutrient concentrations in roots, are more important predictors of aspen seedling outplanting performance than stem traits, with a RSR over 2.0 and height under 40 cm shown to be thresholds for increased outplanting success (Martens et al. 2007; Landhäusser et al. 2012a, b). The only available nursery protocol specific to aspen focuses on maximizing root provisioning through the induction of premature bud set using a hormonal shoot growth inhibitor (SGI) during the growing season, and allowing continued photosynthesis and fertilization (Landhäusser et al. 2012a; Schott et al. 2013). Other methods for inducing bud set were tested (i.e. blackout treatments and nutrient reduction), though the SGI was the most effective (Landhäusser et al. 2012a). However, while this protocol is effective for boreal aspen seed sources, it has not been tested on seed sources from other regions like the US Intermountain West, where there is growing interest in planting aspen for ecological restoration. In this study we investigated whether the nursery protocol developed by Landhäusser et al. (2012a), developed exclusively with boreal aspen seed sources and commercially applied in tree nurseries, would produce similar

desirable traits in aspen seedlings sourced from two populations in the southwestern portion of the species range.

Materials and methods

Seedling nursery production

Aspen seed used in this study was collected in three geographically separate regions in the late spring of 2014. In all three areas, seeds were collected from multiple open-pollinated clones. The two southwestern seed sources were collected in Utah and New Mexico, USA, while the boreal source was collected in Alberta, Canada. The Utah seeds were collected from 15 separate clones (min 0.4 km, max 13 km distance) in Logan Canyon, Utah, USA (N 41°56′; W 111°31′). The New Mexico seeds were collected from 7 separate clones (min 6.4 km, max 127 km distance) near Taos, New Mexico, USA (N 36°24′; W 105°34′). The Alberta source was collected from 15 separate clones (min 1.2 km, max 15 km distance) near Edmonton, Alberta, Canada (N 56°43′; W 113°31′) in the dry parkland region (i.e. the boreal forest—prairie ecotone). This and other Alberta sources are already known to respond well to the nursery protocol suggested by Landhäusser et al. (2012a) which is currently being used in commercial tree nurseries in Alberta to grow over 1 million aspen seedlings per year. Each of the three seedlots represented an approximately equal mixture of seeds from each clone. Seeds were stored in a −20 °C freezer following collection, extraction, and drying. A cursory germination test of 100 seeds per source at the time of sowing showed consistently high germination across sources (> 90%).

Starting in the spring of 2015, aspen seedlings were grown at the New Mexico State University John T. Harrington Forestry Research Center in Mora, NM. D16 Deepot® cells (Stuewe and Sons, Inc., Tangent, OR, USA) with cavities 17.8 cm deep and 5 cm in diameter (262 mL) were filled with a 2:1:1 mixture of sphagnum peat moss, vermiculite, and Turface® clay granules, respectively. Prior to sowing, the media was irrigated until saturation and then misted with 100 g/19 L of 10–30–20 NPK fertilizer. Cells were hand-seeded in the second week of March, each containing three or more aspen seeds from one of the three sources described above. Racks holding 50 cells each were separated by seed source and distributed randomly throughout the greenhouse. For 3 weeks after sowing, the seedlings were misted for 3–5 min, five times per day. At the end of 3 weeks, cells were thinned to one seedling per pot, and the irrigation schedule switched to a target dry-down of 85% of media field capacity. Weekly fertigation began at this time with a 10–52–10 NPK solution (200 ppm), switching to a 15–30–15 NPK solution (200 ppm) after the first 2 weeks. Greenhouse conditions were maintained at 21–24 °C during the day and 17–21 °C at night. After 7 weeks the temperature was changed to maintain a daily range of 15–18 °C. Average humidity was 65% and high-intensity metal halide lamps were used to extend the photoperiod to 14 h.

Seedlings were moved to sub-irrigation tanks in a partial greenhouse (i.e., roof only) 9 weeks after germination when the natural photoperiod reached 14 h, where they continued to receive the same schedule of watering and fertigation. At week 11, all seedlings were treated with an initial application of the shoot growth inhibitor (SGI) paclobutrazol (Bonzi®, Syngenta, Wilmington, DE, USA) at a concentration of 20 mg L^{-1} by adding 5 mL Bonzi® per liter to the sub-irrigation tank and allowing the seedlings to soak in the solution for 15 min before draining. Due to a lack of bud set response in the Utah and New

Mexico sources, a second treatment with Bonzi® was applied to all sources at week 14 following the above procedure. At week 18 the weekly fertilization was increased to 300 ppm of 15–30–15 NPK fertilizer for 2 weeks before increasing one final time to 400 ppm for 2 weeks. Fertilization was discontinued after week 21 and the watering schedule was changed to a target dry-down of 75% of media field capacity at week 22 until seedlings naturally reached dormancy. Additionally, due to partial leaf defoliation of aspen seedlings by cottonwood leaf beetles (*Chrysomela scripta* Fabricius) at the end of June, a 2:1 mixture of organic Insecticidal Soap (15.6 mL soap per L water) and End All® insecticide (no dilution; 1.912% active ingredient, Safer® Brand, Lititz, PA, USA) was hand-sprayed on all seedlings 1–2 times per week as needed until the end of August for a total of 9 applications.

Seedling measurements

To assess development of seed sources over time, there were two destructive sampling periods: 12 weeks after sowing (June; 1 week after the first SGI application) and 35 weeks after sowing (November; fully dormant seedlings). At the first sampling period 30 randomly subsampled seedlings per source were measured for height, root collar diameter (RCD), root, shoot, and leaf dry weights, and terminal bud set. After recording seedling height and RCD, a leaf was removed from the same position on each seedling and digitally scanned to calculate leaf area using the program ImageJ (Schneider et al. 2012). This leaf was then dried and weighed in order to calculate the specific leaf area (SLA, the ratio of leaf area to mass). Seedlings were removed from their containers and the excess media was carefully washed from the root systems. Root volume was measured using the water displacement method (Burdett 1979). The stems were cut at the root collar before drying the stem, root, and remaining leaf tissue of all destructively harvested seedlings in a 70 °C oven for 48 h. Dry weights of these tissues were used to calculate the root-to-stem ratio (RSR) and total leaf dry weight. Due to substantial foliar damage by insects (predominantly cottonwood leaf beetles) and fungi (predominantly Marssonina) during the midsummer, which appeared to differ in severity by source, we performed a visual assessment of all racks of aspen seedling stock and quantified the percentage of damaged leaves per rack by source and damage type.

At the second sampling period (November), 30 randomly selected seedlings per source were selected to assess final seedling characteristics. Seedling height, RCD, root volume, root and stem dry weights were assessed, and terminal bud volumes were estimated from measurements of the length and diameter of the bud (volume calculated as an ellipsoid). Concentrations of nitrogen (N), phosphorus (P), potassium (K), and non-structural carbohydrate (NSC, the sum of soluble sugars and starch) in root and stem tissues were determined. Root and stem tissue samples from each seedling were separated and dried at 70 °C for 48 h before grinding them through a 40-mesh (0.4 mm) screen with a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). N concentrations were assessed using the Kjeldahl method (Kalra and Maynard 1991). P and K concentrations were analyzed using inductively-coupled plasma optical emission spectrometry (ICP-OES) following microwave digestion (EPA Method 3051, U.S. Environmental Protection Agency, Washington, D.C.). NSC concentrations were determined by extracting water soluble sugars three times from each sample with 80% ethanol at 95 °C. Total sugar concentration in the ethanol extract was analyzed using the standardized phenol–sulfuric acid (Landhäusser et al. 2018). Starch content was determined from the residue remaining after sugar extraction using α-amylase and amyloglucosidase digestion followed by the colorimetric

measurement of the glucose hydrolysate using a peroxidase-glucose oxidase-o-dianisidine reagent (Landhäusser et al. 2018).

Statistical analyses

To assess differences in seedling responses to the nursery protocol among the three aspen seed sources, we analyzed response variables with a one-way analysis of variance (ANOVA), and then used a post hoc Tukey's test to evaluate pairwise comparisons between specific sources for statistical significance. Statistical significance was determined at $\alpha = 0.05$ and all ANOVAs were conducted using R 3.4.1 (R Core Team 2017) with packages "multcomp" (Hothorn et al. 2008) and "lsmeans" (Lenth 2016).

Results

Growth differences among the aspen seedling sources were already apparent by the first destructive measurements taken in early June (12 weeks after sowing). The New Mexico source favored early above-ground growth, producing seedlings with the greatest average height, RCD, stem dry weight, and total leaf dry weight ($P < 0.001$ for all), but the lowest root volume and root dry weight ($P < 0.001$ for both) (Table 1). The Alberta source displayed the opposite trend, producing seedlings with the largest roots and the smallest stems and leaf dry weights. Seedlings from the Utah source exhibited intermediate growth, always falling between the New Mexico and Alberta seed sources for these traits. As a result of these growth differences, the RSR of each seedling source differed significantly, with New Mexico at 1.0, Utah at 1.8, and Alberta more than double either of the other sources at 3.8 ($P < 0.001$) (Table 1). The proportion of bud set also varied by seedling source, with 63% bud set in Alberta seedlings compared with 23% in Utah and only 3% in New Mexico. Additionally, susceptibility to foliar fungal infection (primarily *Marssonina*) differed significantly among sources, with an estimated 30% ($SD = 33\%$) of Utah seedling leaves affected, but only 7% ($SD = 2\%$) of New Mexico and 11% ($SD = 5\%$) of Alberta affected. Foliar insect herbivory estimates (primarily *Chrysomela scripta*) were significantly lower in the Alberta source (6%, $SD = 6\%$) compared to New Mexico source (9%, $SD = 4\%$), but did not differ significantly from Utah seedlings (7.4%, $SD = 5\%$).

Between the June and November sampling, seedling sources responded differently to the SGI applications (Fig. 1). In the Alberta source, nearly all seedlings set bud and terminated additional shoot growth for the rest of the growing season. In contrast, while most of the Utah and New Mexico seedlings terminated shoot growth initially following the SGI treatments, the majority broke bud (terminal and/or axillary) or suckered from the roots and continued to grow new stem and leaf tissue late into the growing season.

Results from the November sampling showed an even greater disparity between sources with respect to RSR. Alberta seedlings possessed a RSR of 9.7, nearly three times greater than Utah seedlings (3.4) and more than four times greater than New Mexico seedlings (2.3) (Table 2). Differences in RSR between sources were largely driven by stem size variation, with seedling height and RCD differing significantly between all sources ($P < 0.001$ for both). Stem size followed the same trend as the previous measures in early June, with New Mexico seedlings having the largest stems and Alberta the smallest (Table 2). Final average root dry weights were similar between Alberta and New Mexico (2.6 g and 2.5 g respectively), but both were still significantly greater than Utah (1.8 g, $P = 0.003$) (Table 2).

Table 1 Mean (standard deviation) morphological responses observed in seedling stock from first destructive subsampling (June 2, 2015, 12 weeks after sowing)

Seed source	Height (cm)	RCD (mm)	Root volume (mL)	Root dry weight (g)	Stem dry weight (g)	Root:stem ratio	Specific leaf area (cm^2/g)	Total leaf dry weight (g)
Alberta	11.8 a (3.3)	1.9 a (0.4)	3.4 a (0.9)	0.67 a (0.21)	0.19 a (0.08)	3.8 a (1.1)	181 a (23)	0.33 a (0.11)
Utah	19.2 b (5.1)	2.1 a (0.3)	3.1 a (0.7)	0.54 b (0.17)	0.32 b (0.11)	1.8 b (0.6)	183 a (22)	0.47 b (0.12)
New Mexico	29.1 c (7.0)	2.4 b (0.4)	2.6 b (0.6)	0.41 c (0.12)	0.43 c (0.15)	1.0 c (0.4)	203 b (22)	0.58 c (0.14)

Different letters connote significant differences between seed sources (n = 30)

Fig. 1 Representative nursery-grown aspen seedlings from **a** Utah, **b** New Mexico, and **c** Alberta 2 months after sowing

Final seedling nutrient and NSC tissue concentrations also differed between seedling sources. Whole-plant N concentration was significantly higher in the Alberta seedlings (48.0 mg/g) than in the Utah and New Mexico seedlings (37.5 mg/g and 33.4 mg/g, respectively, $P < 0.001$) as was P concentration (6.0 mg/g in Alberta seedlings, 4.6 mg/g in both Utah and New Mexico seedlings, $P < 0.001$) (Table 3). Only K concentration did not differ between sources ($P = 0.183$) (Table 3). Stem NSC concentration was greatest in the Alberta

Table 2 Mean (standard deviation) morphological responses observed in seedling stock from final destructive subsampling following seedling dormancy (November 6, 2015, 35 weeks after sowing)

Seed source	Height (cm)	RCD (mm)	Root volume (mL)	Root dry weight (g)	Stem dry weight (g)	Root:stem ratio	Bud volume (mm^3)
Alberta	13.0 a (4.7)	3.2 a (0.6)	12.4 ab (4.0)	2.6 a (1.0)	0.32 a (0.19)	9.7 a (3.5)	12.3 a (4.8)
Utah	20.4 b (8.3)	3.7 b (0.9)	10.0 a (3.7)	1.8 b (0.8)	0.62 a (0.42)	3.4 b (1.1)	12.6 a (5.4)
New Mexico	34.5 c (12)	4.8 c (0.9)	13.2 b (4.4)	2.5 a (1.0)	1.35 b (0.76)	2.3 b (1.4)	11.4 a (3.8)

Different letters connote significant differences between seed sources (n = 30)

Table 3 Average (standard deviation) of nutrient and carbohydrate reserves measured in seedling stock from final destructive subsampling following seedling dormancy (November 6, 2015, 35 weeks after sowing)

Seed source	N (mg/g)	P (mg/g)	K (mg/g)	Root NSC (%)	Root NSC (g)	Stem NSC (%)	Stem NSC (g)
Alberta	48.0 a (5.6)	6.0 a (0.57)	8.6 a (1.2)	33.3 a (2.8)	0.84 a (0.33)	16.2 a (2.7)	0.06 a (0.03)
Utah	37.5 b (5.8)	4.6 b (0.53)	8.7 a (1.4)	23.4 b (10.0)	0.48 b (0.31)	13.2 b (3.7)	0.10 b (0.06)
New Mexico	33.4 c (7.0)	4.6 b (0.53)	8.0 a (0.8)	28.0 ab (6.3)	0.65 ab (0.35)	12.9 b (1.6)	0.14 b (0.08)

Different letters connote significant differences between sources (NSC: n = 20; Alberta NPK: n = 17; New Mexico NPK: n = 20; Utah NPK: n = 19)

NSC = total non-structural carbohydrates (sugars + starch)

NPK are whole plant values

seedlings ($P < 0.001$), although these levels only differed significantly from the Utah source ($P < 0.001$) (Table 3). Root NSC (%) was the least variable in Alberta seedlings (Table 3).

Discussion

Aspen seedlings sourced from the three geographically distinct regions differed markedly in their response to the nursery protocol, producing seedlings with significantly different morphological traits, as well as nutrient and carbohydrate reserves. Differences between the boreal Alberta source and the US Intermountain West sources from Utah and New Mexico are not particularly surprising given the latitudinal difference among seed source origins and the phylogeographic boundary known to exist between aspen populations in the US Intermountain West and elsewhere throughout their range (Callahan et al. 2013). Our findings suggest that additional optimizations of the nursery protocol are necessary, and that a direct comparison of this protocol to other protocols would be useful for this wide-ranging species. More unexpected were the differences we observed between the Utah and New Mexico seedlings, given that no differences were previously observed between seed sourced from various places in Alberta used during development of the nursery protocols (Martens et al. 2007; Landhäusser et al. 2012a, b; Schott et al. 2013). In particular, New Mexico seedlings grew significantly larger than Utah seedlings in most metrics, developing greater height, RCD, root volume, and root dry weight (Table 2). Utah seedlings, however, developed a greater tissue concentration of N (Table 3), and while not statistically different, a 48% greater RSR than New Mexico seedlings (Table 2).

Interestingly, Utah seedlings also experienced fungal leaf infection rates more than four times greater than those observed in New Mexico seedlings. Paclobutrazol inhibits growth by reducing the biosynthesis of gibberellins (GA), which are diterpenes (Rademacher 2000). It is possible that the Bonzi® treatments reduced the biosynthesis of other terpenoids important in defense against both insects and fungi, and this effect could vary by source.

The significant difference in seed source response suggests the importance of considering seed source based not only on outplanting location, but also on how sources will

perform in the nursery. Moreover, it indicates that local adaptation of nursery protocols to optimize aspen seedling stock quality will likely be necessary in the US Intermountain West, where significant genetic population structuring by geographic distance exists (Callahan et al. 2013). Testing additional seed sources from across aspen's range will be an important next step to determine the consistency of response to nursery protocols both regionally and sub-regionally. Within-source variation (e.g. among clones or maternal trees within clones) may also contribute to seedling variability in response to protocols. Aspen in the US Intermountain West is known to have dramatic phenotypic and physiological variability among clones within a population (e.g. Kanaga et al. 2008). Seedling characteristics could also be influenced by maternal effects, although the low maternal investment in aspen seeds may minimize variation within clones.

Differences in growth among seed sources were likely influenced by the SGI application. Although the SGI was intended to induce early bud set, most seedlings from both the Utah and New Mexico sources reflushed or suckered from the root system following application and continued shoot growth later into the growing season. The Alberta seedlings, by contrast, responded to the nursery protocol as expected (Landhäusser et al. 2012a, b; Schott et al. 2013); uniformly setting and holding bud throughout the rest of the growing season, leading to the development of higher RSR, root and shoot NSC, as well as whole-plant woody tissue concentrations of N and P compared to the Utah and New Mexico sources. Though higher latitude populations tend to have a longer critical photoperiod and set bud earlier when moved toward the equator (e.g. Luquez et al. 2008), it is unlikely that this factor would have caused early bud set in the Alberta seedlings in the absence of the SGI treatment. Aspen has an indeterminate growth strategy and Alberta seedlings naturally set bud in early September when day length is still approximately 13 h in Edmonton, but night temperatures are approaching freezing (Simon Landhäusser, personal observation). Our nursery protocol maintained a 14-h photoperiod following germination and seedlings were only moved outside once the natural day length reached > 14 h.

Despite the inconsistent bud set responses of the Utah and New Mexico seedlings to the protocol, the SGI treatment still appeared to have a marked effect on overall seedling growth. Although this study did not directly compare seedlings grown without a SGI treatment, a subsequent study unrelated to this experiment grew the same three seed sources without the use of a SGI in a greenhouse setting with smaller containers (164 mL cells), but under otherwise similar conditions. These aspen seedlings had 79%, 68%, and 70% lower RSRs and 23%, 33%, and 61% lower average root NSC concentrations for Alberta, Utah and New Mexico seedlings, respectively, compared to our study (Owen Burney, unpublished data). These differences suggest that the SGI treatment was at least partially effective in its goal of increasing RSR and NSC. It is also notable that all sources developed a final RSR well over 2.0, and a height under 40 cm, which are traits associated with improved aspen seedling quality in marginal environments (Landhäusser et al. 2012a). Nevertheless, research targeting protocol optimization for particular seed sources will need to explore the most effective timing and concentration for SGI application to produce a more consistent bud set.

In addition to refining the SGI application, testing alternative methods for increasing RSR could also be explored. Artificially shortening day length through the temporary application of a blackout treatment midway through the growing season has also been effective in the induction of early bud set in aspen, but only when seedlings are grown outdoors (Landhäusser et al. 2012a). A blackout treatment has the potential to be a lower cost alternative to the application of a SGI, however in experiments with Alberta aspen seed sources, bud set was shown to be highly variable in response to this treatment (Landhäusser

et al. 2012a). This approach has not been tested in other aspen seed sources. Additionally, top-pruning seedlings to a standardized height prior to outplanting has been demonstrated to increase establishment success of many hardwood tree species in marginal environments (South 1996), though removal of stem tissue could have energetic costs to subsequent seedling growth, particularly in areas with limiting site conditions.

Our results indicate that the aspen seedling production protocol we used—which is an effective tool to increase aspen seedling quality in the boreal forest region—will require more adaptations for its use with aspen in the US Intermountain West. While the overall quality of a seedling is ultimately defined by performance at the outplanting site, nursery protocols play a significant role and their development is an iterative process that can take decades to refine. This process only began recently for aspen as its potential in forest reclamation has become recognized (Landhäusser et al. 2019). In the US Intermountain West, aspen reforestation and afforestation using planted seedlings is not yet widely practiced. Natural sexual regeneration appears to be less common in this region than it is in the eastern US and Canada (Landhäusser et al. 2019), and asexually regenerated clones limit genetic diversity and thus resiliency to climate change and other environmental stressors (Gray et al. 2011; Long and Mock 2012). Considering these factors and the increasing need for aspen restoration following severe fires, we anticipate a growing demand in the US Intermountain West for nursery-produced seedlings to maintain aspen at the landscape scale.

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